University of Alberta

BIODEGRADATION OF METABOLITES FROM CONDENSED THIOPHENES FOUND IN PETROLEUM

by

DAVID CURTIS BRESSLER

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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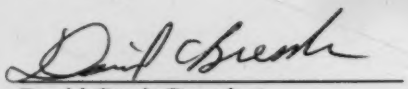

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To my wife, parents, and mentors,

who selflessly gave of themselves,

to allow me to build a better person of myself.

ABSTRACT

Condensed thiophenes comprise a small but significant portion of fossil fuels and their derivatives. Analysis of samples recovered from environments contaminated with petroleum or fossil fuel derivatives frequently demonstrates the persistence of these compounds. Representative model compounds include benzo[*b*]thiophene and dibenzothiophene. Previous work has shown the loss of these compounds resulting in the formation of oxidized metabolites. This study focussed on the aerobic biodegradation of some of these condensed thiophene metabolites. Compounds studied included both biodegradation metabolites of dibenzothiophene (including benzothiophene-2,3-dione and 3-hydroxy-2-formylbenzothiophene) and metabolites of benzothiophene and methyl benzothiophenes (benzothiophene sulfone, 3- and 5-methylbenzothiophene sulfones and benzothiophene-2,3-dione).

Pseudonocardia strain DB1 was isolated based upon its ability to utilize benzothiophene sulfone as sole carbon, energy and sulfur source. It was shown to utilize 3- and 5-methylbenzothiophenes as sole carbon, energy and sulfur sources as well as metabolize the analog 5-fluorobenzothiophene sulfone when grown on benzothiophene sulfone. Strain DB1 mineralized the sulfones demonstrating large amounts of carbon dioxide, sulfate, and fluoride release. 2,3-Dihydro-2-hydroxybenzothiophene sulfone was observed as a metabolite of benzothiophene sulfone and 5-fluoro-2,3-dihydro-2-hydroxybenzothiophene sulfone was identified as a metabolite of 5-fluorobenzothiophene sulfone. Benzonaphthothiophene sulfone was also observed as an abiotic condensation product of benzothiophene sulfone.

This dissertation revealed that benzothiophene-2,3-dione and 3-hydroxy-2-formylbenzothiophene were both chemically unstable and were observed to undergo condensation reactions. At neutral pH, benzothiophene-2,3-dione undergos an abiotic ring opening to form 2-mercaptophenylglyoxylate. This open ring compound was observed to under go a sulfur oxidation to yield a large disulfide compound which subsequently decarbonylated twice, with observable intermediates, to form 2,2'-dithiosalicylic acid. Preliminary studies revealed that the 2,2'-dithiosalicylic acid could be biodegraded by a mixed bacterial population.

3-Hydroxy-2-formylbenzothiophene, when protonated, was observed to undergo a condensation reaction resulting in the formation of *cis*- and *trans*-thioindigo. 3-Hydroxy-2-formylbenzothiophene was biodegraded by a mixed bacterial culture, and that benzothiophene-2,3-dione (or 2-mercaptophenylglyoxylate) accumulated in culture supernatants.

The studies described in this dissertation gathered significant evidence that condensation reactions may play a major role in deciding the final fate of carbon and sulfur from condensed thiophenes after biodegradation. It is suggested that future biodegradation investigations seriously consider the search for products with significantly higher molecular weights than starting substrates.

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TABLE OF CONTENTS

1. INTRODUCTION..... 1
   - 1.1 Sulfur heterocycles..... 1
   - 1.2 The condensed thiophenes..... 1
   - 1.3 Types, structures and sources of sulfur heterocycles..... 2
     - 1.3.1 Thiophenes..... 2
     - 1.3.2 Condensed thiophenes..... 3
     - 1.3.3 Thiacycloalkanes..... 4
     - 1.3.4 Penicillins and cephalosporins..... 5
   - 1.4 Biodegradation of sulfur heterocycles..... 6
   - 1.5 Evidence for ring cleavage of condensed thiophenes..... 7
     - 1.5.1 Benzothiophenes..... 7
     - 1.5.2 Dibenzothiophenes..... 9
   - 1.6 Mechanisms of ring cleavage..... 12
     - 1.6.1 Benzothiophene ring cleavage..... 12
     - 1.6.2 Dibenzothiophene ring cleavage..... 13
       - 1.6.2.1 Dibenzothiophene naphthalene-like attack..... 14
       - 1.6.2.2 Dibenzothiophene five-membered ring attack..... 16
       - 1.6.2.3 Dibenzothiophene angular dioxygenase attack..... 19
   - 1.7 Challenges of ring cleavage..... 21
   - 1.8 Research overview and objectives..... 24
   - 1.9 Literature cited..... 26

2. BIODEGRADATION OF BENZOTHIOPHENE SULFONES BY A FILAMENTOUS BACTERIUM..... 35
   - 2.1 Introduction..... 35
   - 2.2 Materials and methods..... 37
     - 2.2.1 Chemicals..... 37
     - 2.2.2 Media..... 37
     - 2.2.3 Biodegradation studies..... 38
     - 2.2.4 Bacterial cultures..... 39
     - 2.2.5 Temperature and pH optima..... 39
     - 2.2.6 Release of inorganic sulfur..... 39
     - 2.2.7 Carbon and sulfur balances in sealed flasks..... 40
     - 2.2.8 Growth substrates..... 40
     - 2.2.9 Analytical methods..... 41
     - 2.2.10 Sequencing the gene encoding the 16S rRNA..... 42
     - 2.2.11 Scanning electron microscopy..... 43
   - 2.3 Results..... 44
     - 2.3.1 Characteristics of strain DB1..... 44
     - 2.3.2 Growth substrates..... 47
     - 2.3.3 Acid production during growth..... 47
     - 2.3.4 Mineralization of the benzothiophene sulfones..... 49
   - 2.4 Discussion..... 52
   - 2.5 Literature cited..... 55

3. *PSEUDONOCARDIA* STRAIN DB1 UTILIZATION OF BENZOTHIOPHENE SULFONE AND SUBSTITUTED BENZOTHIOPHENE SULFONE..... 59
3.1 Introduction..... 59
3.2 Material and methods..... 64
3.2.1 Chemicals..... 64
3.2.2 Synthesis of 5-fluorobenzothiophene sulfone..... 64
3.2.3 Effect of 5-fluorobenzothiophene sulfone on the utilization of benzothiophene sulfone by *Pseudonocardia* strain DB1... 65
3.2.4 Sulfone biodegradation with high cell density of strain DB1.. 65
3.2.5 Analytical methods..... 66
3.3 Results..... 67
3.3.1 Synthesis of 5-fluorobenzothiophene sulfone..... 67
3.3.2 Effect of 5-fluorobenzothiophene sulfone on the utilization of benzothiophene sulfone by *Pseudonocardia* strain DB1... 67
3.3.3 Sulfone utilization in cultures with high cell density of strain DB1..... 71
3.3.4 Identification of sulfone biodegradation metabolties..... 72
3.3.5 Diels-Alder condensation products..... 77
3.4 Discussion..... 79
3.5 Literature cited..... 85

4. PURIFICATION, STABILITY AND MINERALIZATION OF 3-HYDROXY-2-FORMYLBENZOTHIOPHENE, A METABOLITE OF DIBENZOTHIOPHENE..... 88
4.1 Introduction..... 88
4.2 Materials and methods..... 91
4.2.1 Preparation of HFBT by microbiological methods..... 91
4.2.2 Chemical synthesis of HFBT..... 92
4.2.3 Abiotic reactions of HFBT in crystalline form..... 92
4.2.4 Stability of HFBT in sterile aqueous solutions..... 93
4.2.5 Biodegradation and mineralization of HFBT..... 93
4.2.6 Analytical methods..... 94
4.3 Results..... 94
4.3.1 Chemical synthesis of HFBT..... 94
4.3.2 HFBT from culture extracts..... 95
4.3.3 Abiotic transformations of HFBT in crystalline form..... 96
4.3.4 Thioindigo in the supernatant of strain BT1d grown on HFBT 98
4.3.5 Stability of HFBT in sterile aqueous solutions..... 99
4.3.6 Biodegradation and mineralization of HFBT..... 101
4.4 Discussion..... 102
4.5 Literature cited..... 106

5. ABIOTIC REACTION PRODUCTS FROM BENZOTHIOPHENE-2,3-DIONE AND THE BIODEGRADATION OF ONE OF THE PRODUCTS 109
5.1 Introduction..... 109
5.2 Materials and methods..... 111
5.2.1 Chemicals..... 111
5.2.2 Chemical synthesis of benzothiophene-2,3-dione..... 112
5.2.3 Abiotic reactions of benzothiophene-2,3-dione at various pH. 112
5.2.4 Detection and identification of disulfides formed abiotically from benzothiophene-2,3-dione 113
5.2.5 Effect of temperature and light on benzothiophene-2,3-dione stability..... 113
5.2.6 Mixed cultures biodegradation of 2,2'-dithiosalicylic acid.... 113
5.2.7 Isolation of 2,2'-dithiosalicylic acid degraders..... 114
5.2.8 Identification of disulfides as dibenzothiophene biodegradation products..... 115
5.2.9 Quantitative determination of sulfur as high molecular weight biodegradation metabolites..... 115
5.2.10 Analytical methods..... 116
5.3 Results..... 117
5.3.1 Abiotic benzothiophene-2,3-dione degradation products..... 117
5.3.2 Affect of temperature, pH, and light on disulfide formation.. 127
5.3.3 Bacterial disulfide production from dibenzothiophene..... 130
5.3.4 Biodegradation of 2,2'-dithiosalicylic acid..... 131
5.4 Discussion..... 134
5.5 Literature cited..... 141

6. OVERALL DISCUSSION AND SUGGESTIONS FOR FURTHER RESEARCH..... 144
6.1 Overall disscussion..... 144
6.2 Suggestions for further research..... 150
6.3 Literature cited..... 155

A. APPENDIX A. OXIDATION OF CARBAZOLE, N-ETHYLCARBAZOLE, FLUORENE, AND DIBENZOTHIOPHENE BY THE LACCASE OF *CORIOLOPSIS GALLICA* UAMH 8260..... 158
A.1 Introduction..... 158
A.2 Materials and methods..... 159
A.2.1 Enzyme and chemicals..... 159
A.2.2 Laccase assay..... 159
A.2.3 Substate oxidation..... 159
A.2.4 Protein determination..... 160
A.3 Results and Discussion..... 160
A.4 Literature Cited..... 165

LIST OF TABLES

TABLE		PAGE
Table 1.1	Bond strengths in selected compounds.....	23
Table 2.1.	Primers used for the PCR amplification and for sequencing of strain DB1 16S rRNA gene.....	43
Table 2.2.	Alternate carbon sources tested for growth of strain DB1 and *P. compacta* ATCC 35407.....	48
Table 2.3	Sulfate release and carbon balance for strain DB1 utilization of benzothiophene sulfone, 3-methylbenzothiophene sulfone and 5-methylbenzothiophene sulfone.....	51
Table 3.1.	Release of fluoride from 5-fluorobenzothiophene sulfone when incubated with benzothiophene sulfone in sulfate-free mineral salts medium in the presence of *Pseudonocardia* strain DB1.....	69
Table 5.1	Carbon balance for two 6-week-old garden soil cultures grown on 2,2'-dithiosalicylic acid and incubated in triplicate.....	133
Table 6.1	Summary of aerobic studies of microbial metabolism of organosulfur compounds.....	146

LIST OF FIGURES

FIGURE		PAGE
Figure 1.1	Examples of one-, two- and three-ring thiophenes synthesized by plants.....	2
Figure 1.2	Examples of 2,5-dialkylthiophenes that have been identified in bitumens, crude oils and in pyrolysates of kerogens and asphaltenes	3
Figure 1.3	Examples of unsubstituted condensed thiophenes found in petroleum and coal derivatives.....	4
Figure 1.4	Examples of thiolanes and thianes found in non-biodegraded petroleums.	5
Figure 1.5	Penicillin N is converted deacetoxycephalosporin C by an enzyme found in cephalosoporin-producing microorganisms	6
Figure 1.6	2-Mercaptophenylglyoxalate cyclizes under acidic conditions to give benzothiophene-2-3-dione.....	8
Figure 1.7	The "Kodama pathway".....	9
Figure 1.8	The desulfurization of dibenzothiophene by strain IGTS8 using the modified 4S pathway.	11
Figure 1.9	Fluorene, its analogs and their numbering conventions.	13
Figure 1.10	*meta*-Cleavage products after dioxygenase attack at carbons 1 and 2 in dibenzofuran and dibenzothiophene, and carbons 3 and 4 in carbazole and fluorene.....	15
Figure 1.11	Acid catalyzed cyclization of 2'-hydroxybiphenyl-2-sulfonic acid and 2'-hydroxybiphenyl-2-sulfinic acid to form the sultone and sultine respectively	18
Figure 1.12	The pathway for dibenzothiophene metabolism by *Brevibacterium* sp. DO.	20
Figure 1.13	The C—S bond cleavage mechanism for a sulfoxidized hemimercaptal.	21
Figure 1.14	Example of insertion of a metal into the C–S bond of benzothiophene and subsequent hydrodesulfurization.....	22

Figure 2.1	The sequential oxidation of 3-methylbenzothiophene to its sulfone	36
Figure 2.2	Sequencing strategy used to sequence isolate DB1 16S rRNA gene.....	42
Figure 2.3	Morphology of a 21-day-old culture of strain DB1 observed by scanning electron microscopy.....	45
Figure 2.4	Dendrogram of bacteria with 16S rRNA gene sequences similar to that of strain DB1.....	46
Figure 2.5	Changes in the medium during the growth of strain DB1 on benzothiophene sulfone.....	49
Figure 2.6	Changes in the medium during the growth of strain DB1 on 3-methylbenzothiophene sulfone.....	50
Figure 3.1	Benzonaphthothiophene formed through the Diels-Alder condensation of benzothiophene sulfoxide	62
Figure 3.2	Polar oxidized metabolites of benzothiophene and dibenzothiophene	63
Figure 3.3	a) Strain DB1 utilization of benzothiophene sulfone in the presence of various concentrations of 5-fluorobenzothiophene sulfone b) 5-Fluorobenzothiophene sulfone remaining in strain DB1 cultures grown on 100 mg/L benzothiophene sulfone.....	68
Figure 3.4	The degradation of benzothiophene sulfone and 5-fluorobenzothiophene sulfone by strain DB1 at different initial concentrations of the latter sulfone.....	70
Figure 3.5	Degradation of benzothiophene sulfones in cultures with high cell density of *Pseudonocardia* strain DB1.....	71
Figure 3.6	a) GC-MS total ion current and b) mass spectrum of the metabolite produced from benzothiophene sulfone by strain DB1.....	73
Figure 3.7	Infrared spectrum of benzothiophene sulfone strain DB1 biodegradation product obtained through GC-FTIR analysis.....	74

Figure 3.8	Reference mass spectra of (a) phenylethyl alcohol and (b) 1-phenylethanol.....	75
Figure 3.9	GC-MS analysis and mass spectrum of the metabolite produced from 5-fluorobenzothiophene sulfone by strain DB1.....	76
Figure 3.10	GC-MS analysis of benzothiophene sulfone crystals dissolved in dichloromethane	78
Figure 3.11	GC-FTIR spectrum of condensation product formed through a Diels-Alder condensation of two molecules of benzothiophene sulfone	79
Figure 3.12	Biodegradation products of benzothiophene reported by Eaton and Nitterauer (1994).....	83
Figure 4.1	Abbreviated Kodama pathway for DBT biodegradation to HFBT (a) and proposed products from HFBT and references in which they were reported (b).	89
Figure 4.2	Some possible biodegradation products of HFBT and references in which they were reported.....	90
Figure 4.3	Mass spectrum of HFBT.....	95
Figure 4.4	Mass spectrum of two compounds identified as products of abiotic HFBT condensation reactions.....	97
Figure 4.5	Structures of the pink products found with HFBT crystals after 1 year storage.....	98
Figure 4.6	Chromatogram of the dichloromethane extract of a neutral culture supernatant of strain BT1d degrading DBT.....	99
Figure 4.7	Stability of HFBT in various aqueous solutions at 28°C	100
Figure 4.8	UV-visible spectra of the supernatant from a viable enrichment culture growing on HFBT for 1 month and a sterile control.....	101
Figure 5.1.	Equilibrium between benzothiophene-2,3-dione and 2-mercaptophenylglyoxalate.....	110
Figure 5.2	HPLC diode array analysis of benzothiophene-2,3-dione in a pH 11 NaOH solution compared to literature scans.	118

Figure 5.3 Benzothiophene-2,3-dione incubated for 24 h at pH 7 in sulfate-free mineral salts medium 119

Figure 5.4 Positive and negative ionization mass spectra of product C (1.9 min retention time) obtained through HPLC with a mass selective detector..... 120

Figure 5.5 Oxidation of 2-mercaptophenylglyoxylate to a disulfide..... 121

Figure 5.6 Anhydrous sodium sulfate dried acidified extract of benzothiophene-2,3-dione transformation products formed through the incubation of sterile benzothiophene-2,3-dione (100 mg/L) in sulfate-free mineral salts medium at neutral pH (a) Non-dried extract of redissolved sodium sulfate drying agent (b)..... 122

Figure 5.7 Mass spectrum of the three disulfides formed through 2-mercaptophenyl-glyoxalate oxidation and subsequent decarbonylation..... 124

Figure 5.8 Total ion current of authentic 2,2'-dithiosalicylic acid (and thianthrene standard) derivatized with diazomethane (a) mass spectrum of 2,2'-dithiosalicylic acid (b)..... 125

Figure 5.9 HPLC diode array analysis of a 1 month old pH 9 solution of disulfides formed through abiotic degradation of benzothiophene-2,3-dione. a) chromatograph and UV-Vis scan of authentic 2,2'-dithiosalicylic acid standard. b) diode array chromatograph at λ240 nm of solution. c) UV-vis scan of the other two disulfide products..... 126

Figure 5.10 Formation of disulfides from 2-mercaptophenylglyoxalate and subsequent decarbonylation reactions..... 127

Figure 5.11 Effect of pH on disulfide formation as monitored through HPLC analysis at λ240nm of 1-month-old sterile incubations of the 2,3-dione in mineral salts medium. 128

Figure 5.12 Effect of temperature and light exposure on disulfide formation as monitored through HPLC analysis at λ240nm of 18 day old sterile incubations of the 2,3-dione in mineral salts medium..... 129

Figure 5.13 GC-MS total ion current chromatography of the diazomethane derivatized extract of a *Pseudomonas* BT1d culture grown on excess dibenzothiophene in mineral salts medium..... 130

Figure 5.14 Biodegradation of 2,2'-dithiosalicylic acid in mineral salts medium by two mixed bacterial communities (a) and pH change in the cultures (b)..... 132

Figure 5.15 2,2'-Dithiosalicylic acid degrading mixed cultures enriched from garden soil after 6 weeks of incubation in mineral salts medium..... 133

Figure 5.16 Summary of the possible abiotic and biotic reactions leading to the formation of 2,2'-dithiosalicylic acid from dibenzothiophene..... 140

Figure 6.1 Oxidation and condensation reactions of condensed thiophene metabolites..... 149

Figure A.1 GC-MS analyses of the extract of a reaction mixture that contained dibenzothiophene (a,c) and of an authentic standard of dibenzothiophene sulfoxide (b,d)..... 161

Figure A.2 GC-MS analyses of the extract of a reaction mixture that contained fluorene (a,c) and of an authentic standard of 9-fluorenone (b,d) 162

Figure A.3 Summary of results with various substrates in reaction mixtures with laccase. 164

ABBREVIATIONS

HFBT	- 3-hydroxy-2-formylbenzothiophene
HPLC	- high performance liquid chromatography
BSA	- N,O-bis-(trimethylsilyl)acetamide
GC	- gas chromatography
GC-MS	- gas chromatography with a mass selective detector
GC-FTIR	- gas chromatography with a Fourier-transformed infrared detector
HCl	- hydrochloric acid
DBT	- dibenzothiophene
TLC	- thin layer chromatography
PAH	-polycyclic aromatic hydrocarbons
HBT	- 1-hydroxybenzotriazole
ABTS	- 2,2'-azinobis-(3-ethylbenthiazoline-6-sulfonic acid

1. INTRODUCTION*

1.1 SULFUR HETEROCYCLES

Sulfur heterocycles are common constituents of petroleum and liquids derived from coal, and they are found in some secondary metabolites of microorganisms and plants. They exist primarily as saturated rings and thiophenes. Sulfur heterocycles found in the environment often originate from fossil fuels. Spills of petroleum or creosote can lead to contamination of terrestrial and aquatic environments, and evaporation or incomplete combustion of fossil fuels can lead to atmospheric contamination with subsequent deposition in soils and surface waters. For example, the microbially-produced penicillins and cephalosporins have sulfur-containing rings. Some chemicals used in industrial processes also contain sulfur heterocycles. For instance, sulfolane (tetrahydrothiophene sulfone) is used in the Shell Sulfinol™ process for removal of hydrogen sulfide from sour natural gas (Goar 1971; Taylor *et al.* 1991). Spills at sour gas plant sites have caused soil and groundwater contaminations (McLeod *et al.* 1992; Fedorak and Coy 1996; Greene *et al.* 1998).

1.2 THE CONDENSED THIOPHENES

Among the most commonly found sulfur heterocycles are thiophenes. These may have alkyl side chains or may be condensed with one or more benzene ring(s) to form benzothiophenes, dibenzothiophenes, naphthothiophenes or benzonaphthothiophenes. Sulfur is the third most abundant element in crude oils (Speight 1980), and the condensed thiophenes are the most common form in which sulfur is present.

Alkyl dibenzothiophenes have been shown to be persistent in petroleum-contaminated environments (Boehm *et al.* 1981; Hostettler and Kvenvolden 1994; Wang *et al.* 1994) and they concentrate in the tissues of aquatic species (Laseter *et al.* 1981; Ogata and Fujisawa 1985). Nonetheless, C_1- and C_2-dibenzothiophenes are susceptible to

---

* Portions of this chapter have been previously published.
Bressler, D.C., and P.M. Fedorak. 2000. Can. J. Microbiol. 46:397-409.
Bressler, D.C., J.A. Norman, and P.M. Fedorak. 1998. Biodegradation. 8:297-311.

biodegradation (Atlas *et al.* 1981; Fedorak and Westlake 1982, 1984a; Saftić *et al.* 1992, 1993; Hostettler and Kvenvolden 1994; Kropp *et al.* 1997a).

1.3 TYPES, STRUCTURES AND SOURCES OF SULFUR HETEROCYCLES

1.3.1 Thiophenes

Many plants produce thiophene compounds as secondary metabolites, particularly members of the family Asteracea (Compositae) (Christensen and Lam 1990), and dozens of structures have been reported (Bohlmann *et al.* 1973; Christensen and Lam 1990). Plant thiophenes are synthesized by the addition of sulfide or an alkyl sulfide to a conjugated diyne (Schulte *et al.* 1965). The diyne is typically a C_{13} polyyne derived from a fatty acid, such as oleic acid (Christensen and Lam 1990), which allows the formation of thiophenes with one, two or three rings as well as alkyl substituents (Figure 1.1). For example, the three-ring compound, α-terthienyl, was isolated from marigolds (Zechmeister and Sease 1947): its abundance was 15 to 21 mg/kg of fresh marigold petals. Many of these compounds are toxic to other organisms, and may play a role in defense against predators or pathogens (Christensen and Lam 1990). No reports on the natural biodegradation of these plant-derived thiophenes was found.

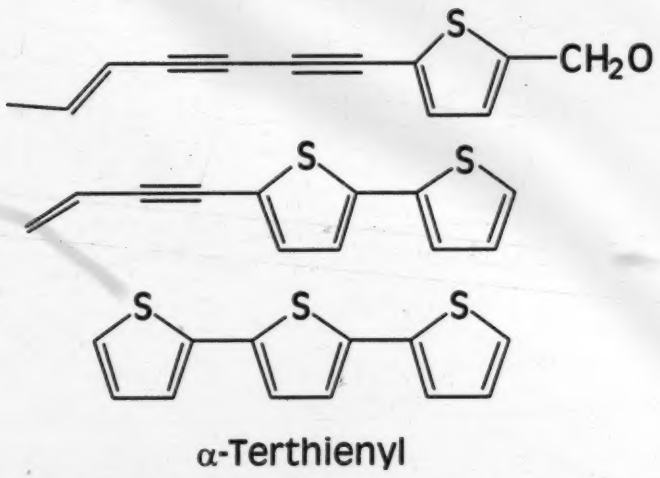


Figure 1.1 Examples of one-, two- and three-ring thiophenes synthesized by plants (after Christensen and Lam 1990).

2,5-Dialkylthiophenes have been identified in bitumens, crude oils and in pyrolysates of kerogens and asphaltenes (Sinninghe Damsté and de Leeuw 1989). Sinninghe Damsté *et al.* (1987, 1989) identified several classes of these, including 2-alkyl-5-methyl-, 2-alkyl-5-ethyl-, 2-alkyl-5-propyl-, and the so called "mid-chain" 2,5-dialkyl- thiophenes. The alkyl substituents may be linear or branched (Sinninghe Damsté *et al.* 1987, 1989) as shown in Figure 1.2.

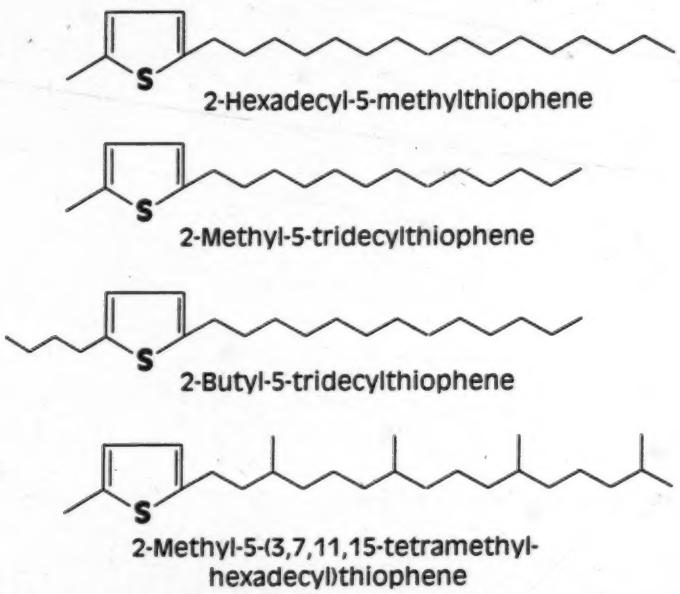
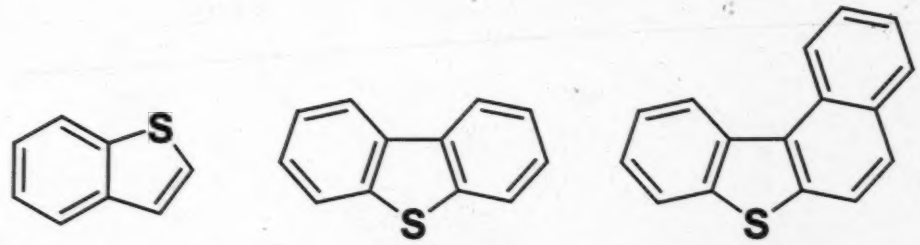


Figure 1.2 Examples of 2,5-dialkylthiophenes that have been identified in bitumens, crude oils and in pyrolysates of kerogens and asphaltenes (after Sinninghe Damsté and de Leeuw 1989).

1.3.2 Condensed thiophenes

The monograph by Jacob (1990) is an excellent reference on structures, sources, synthesis and toxicity of condensed thiophenes. Kropp and Fedorak (1998) published a comprehensive review of the toxicity and biodegradation of condensed thiophenes found in petroleum. Chou (1990) reviewed the forms of sulfur in coal, and among these were condensed thiophenes. Often oxidations, pyrolysis or extractions are required to release

these compounds from the coal. For example, benzothiophene, dibenzothiophene and benzonaphthothiophene (Figure 1.3) were found in bituminous coal and anthracite after oxidation with $Na_2Cr_2O_7$. In addition, C_2- and C_3-benzothiophenes, methyldibenzothiophenes and phenanthrothiophene were tentatively identified in the benzene extracts of a Kentucky high-volatile bituminous coal (Chou 1990).



Benzo[*b*]thiophene Dibenzo[*b,d*]thiophene Benzo[*b*]naphtho[2,1-*d*]-thiophene

Figure 1.3 Examples of unsubstituted condensed thiophenes found in petroleum and coal derivatives (after Jacob 1990).

Czogalla and Boberg (1983) listed about 350 condensed thiophenes found in crude oils. These range in complexity from compounds containing two rings to compounds with nine rings, some of which contain other heteroatoms such as nitrogen and oxygen.

1.3.3 Thiacycloalkanes

Two more classes of sulfur heterocycles found in petroleum and crude oils are thiolanes and thianes (Figure 1.4). The five-member ring of the thiolanes (tetrahydrothiophenes or thiacyclopentanes) is the preferred cyclization product over the six-member ring of thianes (thiacyclohexanes) when synthetic sulfides with linear carbon frameworks are heated in the presence of calcium carbonate to simulate geological conditions (Payzant *et al.* 1989a). The 2,5-dialkylthiolanes occur in significant amounts in immature petroleums (Schmid *et al.* 1987). These cyclic sulfides are more prevalent in petroleums that have not been subjected to biodegradation (Payzant *et al.* 1989a,b; Grimalt *et al.* 1991).

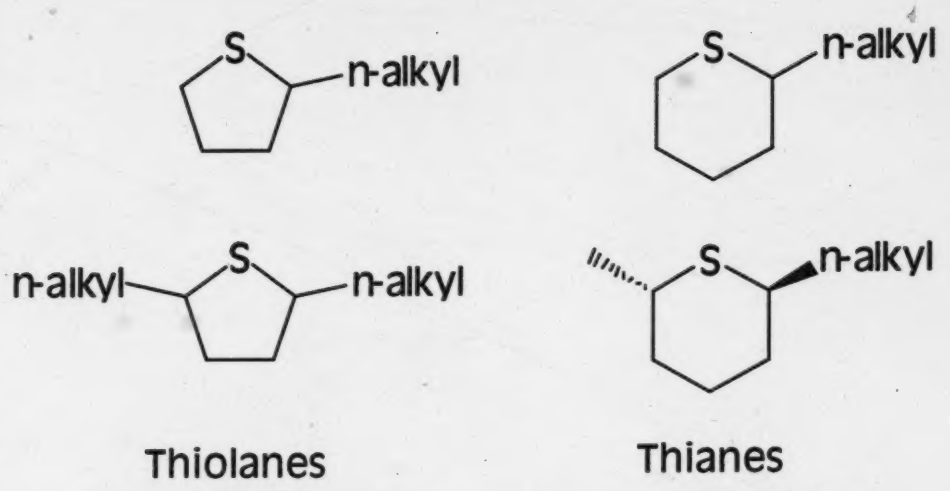


Figure 1.4 Examples of thiolanes and thianes found in non-biodegraded petroleums. The total number of carbon atoms in these compounds ranged from at least C8 to C30 (after Payzant *et al.* 1989a).

Few biodegradation studies have been conducted with these non-aromatic sulfur heterocycles. Using aerobic laboratory cultures, Fedorak *et al.* (1988) demonstrated removal of the side chain of a monoalkylthiolane yielding 2-thiolanecarboxylic acid (2-tetrahydrothiophenecarboxylic acid).

1.3.4 Penicillins and cephalosporins

Penicillins and cephalosporins are important beta-lactam antibiotics synthesized by prokaryotic and eukaryotic microorganisms. Both groups of antibiotics contain two fused rings with one of these containing sulfur and nitrogen atoms. In *Cephalosporium* and *Streptomyces* spp., deacetoxycephalosporin C synthase (or expandase) catalyzes the conversion of the five-member thiazolidine ring (Figure 1.5) in penicillins to a six-member cephem ring in deacetoxycephalosporin C (Baldwin *et al.* 1987). This reaction involves the oxidative expansions of the thiazolidine ring by inserting one of the C2-methyl substituents to give the dihydrothiazine ring of cephalosoporins (Jensen & Demain 1995). In this process, a C–S bond is broken and another C–S bond is formed.

This enzyme requires α-ketoglutarate, Fe^{2+} and O_2 as cofactors, and ascorbate and dithiothreitol for maximum activity (Baldwin *et al.* 1987, Jensen & Demain 1995). Baldwin *et al.* (1991) suggested a mechanism for this expandase activity involving a bridged species intermediate with a sulfur radical or cation which decomposes, with the loss of hydrogen, or introduction of the hydroxyl group derived from α-ketoglutarate-penicillin coupled reduction of dioxygen.

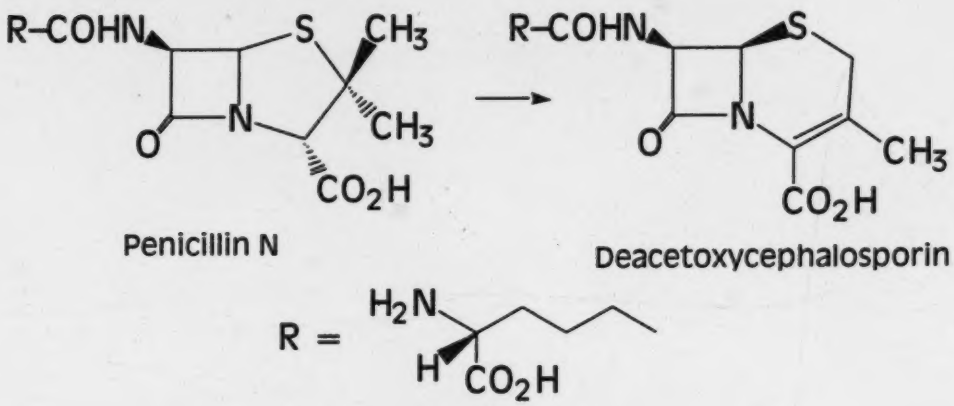


Figure 1.5 Penicillin N is converted deacetoxycephalosporin C by an enzyme found in cephalosoporin-producing microorganisms (after Baldwin *et al.* 1987).

1.4 BIODEGRADATION OF SULFUR HETEROCYCLES

There are two major objectives driving investigations of the microbial metabolism and ring cleavage of condensed thiophenes and other organosulfur compounds in general. One is the quest to develop a process for biodesulfurization of fossil fuels, and the other is to understand the fates of organosulfur compounds in petroleum- or creosote-contaminated environments, which is important in assessing bioremediation processes. In order to understand the environmental fate of a compound, one must first understand which abiotic chemical reactions occur, which potential biodegradation processes are possible, and which biodegradation metabolites are formed. Although it is obviously necessary to determine initial biodegradation products of condensed thiophenes known to exist in petroleum- or creosote-contaminated environments, it is equally as important to determine the further fates of these produced biodegradation products.

Biodegradation and ring cleavage of sulfur heterocycles often results in the release of the sulfur atom from organosulfur compounds, and much of this information has come from investigations focussed on the application of microbial processes for the biodesulfurization of fossil fuels (Monticello and Finnerty 1985; Foght *et al.* 1990; Monticello 1994; Shennan 1996). There is a considerable amount of information on the biodegradation of benzothiophenes (Bohonos *et al.* 1977; Fedorak and Grbić-Galić 1991; Eaton and Nitterauer 1994; Kropp *et al.* 1994a) and dibenzothiophenes (Kodama *et al.* 1970, 1973; Laborde and Gibson 1977; Kargi and Robinson 1984; Monticello *et al.* 1985; van Afferden *et al.* 1990; Saftić *et al.* 1993; Kropp *et al.* 1997a).

1.5 EVIDENCE FOR RING CLEAVAGE OF CONDENSED THIOPHENES

Investigators often look for the release of the sulfur atom from sulfur heterocycles, and this release provides convincing evidence of ring cleavage. The released sulfur atom has been found as sulfide, sulfite and sulfate (Bressler *et al.* 1999; Isbister *et al.* 1988; Kim *et al.* 1990a, 1990b; Kurita *et al.* 1971). In other cases, growth of a culture on a sulfur heterocycle as its sole sulfur source is used as an indication of ring cleavage. A third line of evidence is the identification of some metabolites that contain organosulfur that is not part of a ring system, or the identification of organic metabolites that are devoid of sulfur.

1.5.1 Benzothiophenes

Eaton and Nitterauer (1994) studied the aerobic biotransformation of benzothiophene by isopropylbenzene-degrading bacteria, and detected several metabolites. Among the metabolites that resulted from cleavage of the thiophene, they tentatively identified 2-mercaptophenylglyoxaldhyde, 2'-mercaptomandelate and 2'-mercaptomandelaldehyde, and they unequivocally identified 2-mercaptophenylglyoxalate. Eaton and Nitterauer (1994) observed that the latter compound existed at neutral pH of the culture medium, but it cyclized under acidic conditions to give benzothiophene-2-3-dione (Figure 1.6). This 2,3-dione has been observed in the extracts of acidified cultures incubated with benzothiophene (Bohonos *et al.* 1977; Fedorak and Grbić-Galić 1991; Kropp *et al.* 1994a). Similarly, 2,3-diones were observed in the extracts of acidified

cultures incubated with methyl-substituted benzothiophenes containing the alkyl substituent on the benzene ring (Fedorak and Grbić-Galić 1991; Saftić *et al.* 1992; Kropp *et al.* 1994a). Based on the finding of Eaton and Nitterauer (1994), these 2,3-diones must actually result from cyclization of 2-mercaptophenylglyoxalates which are thiophene ring cleavage products.

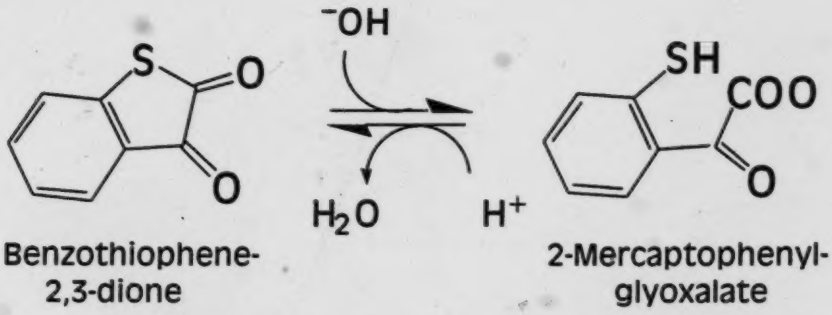


Figure 1.6 2-Mercaptophenylglyoxalate cyclizes under acidic conditions to give benzothiophene-2-3-dione (after Eaton and Nitterauer 1994).

Interestingly, photooxidation of benzothiophene gave benzothiophene-2,3-dione (Andersson and Bobinger 1992). 2-Mercaptophenylglyoxalate was postulated to form from the dione, and subsequent photooxidations of the former compound oxidized the sulfhydryl group to a sulfonic acid, and caused the lost CO to give an 85% yield of 2-sulfobenzoic acid.

Saftić *et al.* (1992) identified *m*-tolyl methyl sulfoxide as an aerobic bacterial metabolite of 6-methylbenzothiophene, and Kropp *et al.* (1994a) identified *o*-tolyl methyl sulfoxide as a metabolite of 7-methylbenzothiophene. These products can only be formed as a result of cleavage of the heterocyclic ring, but the mechanism of their formation is unknown.

Sulfones have been found as bacterial metabolites of various benzothiophenes (Fedorak and Grbić-Galić 1991; Saftić *et al.* 1992; Selifonov *et al.* 1996). A bacterial culture that uses benzothiophene sulfone as its sole carbon and sulfur source has been described (Bressler *et al.*, 1999) and will be discussed later. In addition, sulfate was observed to accumulate in this culture, evidence of further metabolism of the sulfone to release sulfate.

There are some reports of anaerobic biodegradation of benzothiophene leading to cleavage of the thiophene ring. Using bacteria in oil sludge, Kurita *et al.* (1971) observed hydrogen sulfide release from benzothiophene, but no identification of the resulting organic compounds was given. Grbić-Galić (1989) described a study in which benzothiophene was degraded in methanogenic microcosms containing aquifer solids and water from a creosote-contaminated aquifer. Gas chromatography-mass spectrometry was used to identify some of the metabolites, and some of these were *o*-hydroxybenzenesulfonic acid, phenylacetic acid, benzoic acid and phenol.

1.5.2 Dibenzothiophenes

The first reported pathway for dibenzothiophene biodegradation was the so-called "Kodama pathway" (Figure 1.7) (Kodama *et al.* 1970, 1973). It involves oxidation of one of the benzene rings, yielding 3-hydroxy-2-formylbenzothiophene as the most commonly identified product. Further oxidation of this metabolite may yield benzothiophene-2,3-dione, which was found by Bohonos *et al.* (1977) in extracts of cultures incubated with dibenzothiophene.

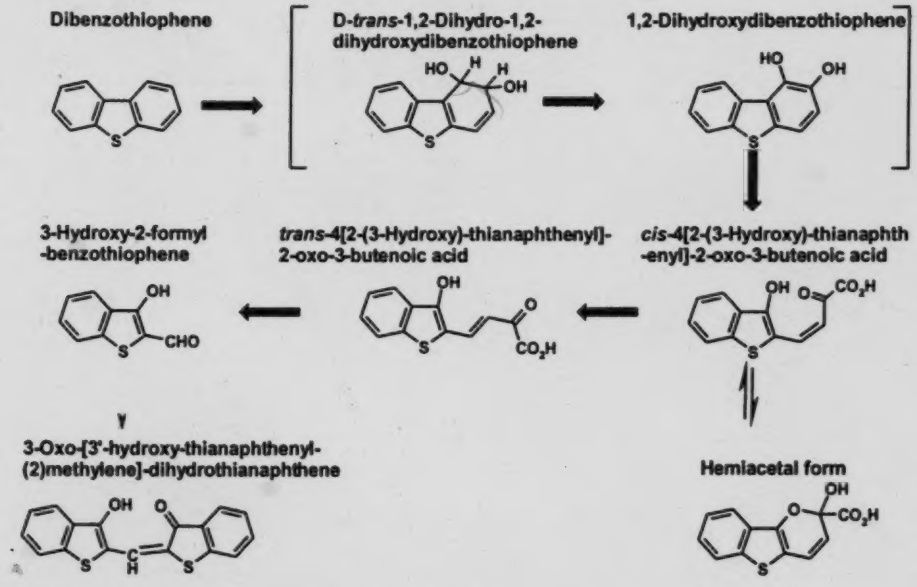


Figure 1.7 The "Kodama pathway" (Kodama *et al.* 1970, 1973)

In light of the results of Eaton and Nitterauer (1994) (Figure 1.6), the 2,3-dione must result from cyclization of the ring cleavage product, 2-mercaptophenylglyoxalate. In other laboratory studies, there have been numerous 2,3-diones found in extracts from acidified cultures incubated with dibenzothiophenes. For example, benzothiophene-2,3-dione was found in the extracts of acidified cultures of three stains of *Pseudomonas* incubated with dibenzothiophene (Kropp and Fedorak 1998). Similarly, the corresponding isomers of methylbenzothiophene-2,3-dione were found as metabolites of each of the four isomers of methyldibenzothiophene (Saftić *et al.* 1993). Also, 4,6-dimethylbenzothiophene-2,3-dione and 4,7-dimethylbenzothiophene-2,3-dione were identified in cultures incubated in the presence of 4,6- and 4,7-dimethylbenzothiophene, respectively (Kropp *et al.* 1996). In addition, 6,7-dimethylbenzothiophene-2,3-dione was identified as a metabolite of 3,4-dimethyldibenzothiophene, and 5-methylbenzothiophene-2,3-dione was a metabolite of 2,8-dimethyldibenzothiophene (Kropp *et al.* 1997a). Thus, the metabolism of several dibenzothiophenes yield 2,3-diones that exist as 2-mercaptophenylglyoxalates at the neutral pH of the culture medium (Figure 1.6).

Kim *et al.* (1990a, 1990b) investigated the potential of anaerobic biodesulfurization by sulfate-reducing bacteria. Under a hydrogen atmosphere, their cultures transformed dibenzothiophene to biphenyl and released hydrogen sulfide.

Pioneering work by Isbister *et al.* (1988) showed that the sulfur atom from dibenzothiophene could be selectively removed from this molecule. Using radiolabeled dibenzothiophene and a genetically modified bacterium "CB1" they demonstrated the liberation of ^{35}S-sulfate from ^{35}S-dibenzothiophene. Using ^{14}C-dibenzothiophene, Isbister *et al.* (1988) found no $^{14}CO_2$ liberation and no incorporation of ^{14}C into biomass of CB1. The bacterium produced 2,2'-dihydroxybiphenyl as an unassimilated organic residue.

This mode of enzymatic attack became known as the "4S pathway" (Krawiec 1990), in which bacteria selectively oxidize the S atom in dibenzothiophene to the sulfoxide, the sulfone, sulfonate, and release it as sulfate, with no cleavage of C–C bonds, thereby maintaining the caloric value of the hydrocarbon moiety of dibenzothiophene. Other investigators have reported that the carbon atoms in dibenzothiophene are released as

biphenyl or monohydroxybiphenyl, and Krawiec (1990) refers to the pathway that yields these products as the "modified 4S" or "extended 4S" pathway.

The isolation and characterization of *Rhodococcus rhodochrous* IGTS8 (Kilbane and Jackowski 1992; Kayser *et al.* 1993) led to major advancements in the investigations of biodesulfurization. Work with this bacterium has provided insight into the mechanism of the thiophene ring cleavage. Indeed, Energy Biosystems Corporation was reported to be using a 5-barrel/day pilot plant to study the biodesulfurization of diesel fuel (Rhodes 1995) by *R. rhodochrous* IGTS8, which was shown to be a strain of *Rhodococcus erythropolis* by 16S rRNA and physiological studies (Monticello *et al.* 1995). Figure 1.8 shows desulfurization of dibenzothiophene by strain IGTS8 using the modified 4S pathway.

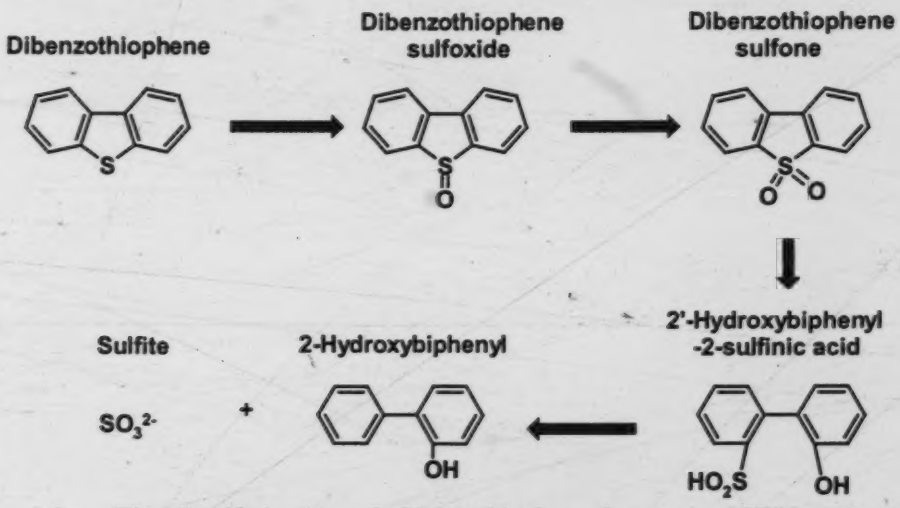


Figure 1.8 The desulfurization of dibenzothiophene by strain IGTS8 using the modified 4S pathway (after Gray *et al.* 1996).

The versatility of organisms using the modified 4S pathway has been examined in several studies. *Rhodococcus* spp. can desulfurize many condensed thiophenes (Monticello 1994), including benzo[*b*]naphtho[1,2-*d*]thiophene (Ohshiro *et al.* 1996). Lee *et al.* (1995) demonstrated that an *Arthrobacter* species could desulfurize the sterically hindered compound 4,6-diethyldibenzothiophene, yielding 2-hydroxy-3,3'-diethylbiphenyl as the sulfur-free product. Similarly, Ohshiro *et al.* (1996) showed that *R. erythropolis* H-2 removed the sulfur atom from 2,8-dimethyldibenzothiophene, 4,6-

dimethyldibenzothiophene and benzo[*b*]naphtho[2,1-*d*]thiophene. The product from the desulfurization of the latter compound was identified as α-hydroxy-β-phenylnaphthalene by gas chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy. The paper by Ohshiro *et al.* (1996) appears to be the first evidence of microbial attack of a four-ring condensed thiophene.

There have been reports of bacterial oxidation of the sulfur in dibenzothiophene and subsequent degradation of the homocyclic rings. For example, van Afferden *et al.* (1990, 1993) describe *Brevibacterium* sp. DO that uses dibenzothiophene as its sole source of sulfur, carbon and energy. Similarly, an aerobic microorganism isolated from a deep sea thermal vent utilized dibenzothiophene as its sole sulfur and carbon source, but grew better in medium supplemented with yeast extract (Kitchell *et al.* 1991).

Using the bacterial isolate FE-9 suspended in dimethylformamide, Finnerty (1993) demonstrated that dibenzothiophene was desulfurized to biphenyl and hydrogen sulfide under an atmosphere of nitrogen or hydrogen. The highest conversion rates were under a hydrogen atmosphere. When incubated with air in the headspace of the reaction tube, isolate FE-9 produced a mixture of biphenyl, 2-hydroxybiphenyl, 2,2'-dihydroxybiphenyl and sulfate from dibenzothiophene.

1.6 MECHANISMS OF RING CLEAVAGE

1.6.1 Benzothiophene ring cleavage

Although benzothiophene and substituted benzothiophenes have been studied in some detail, mechanisms of ring cleavage have not been identified. The presence of metabolites such as 2-mercaptophenylglyoxylate, tolyl methyl sulfoxides, 2-mercaptophenylglyoxaldehyde, 2'-mercaptomandelate and 2'-mercaptomandelaldehyde indicates that ring cleavage has occurred, but the exact mechanism remains unknown. Most reports indicate that benzothiophenes can be cometabolized but cannot serve as sole carbon and energy sources for microorganisms (Sagardía *et al.* 1975; Bohonos *et al.* 1977; Fedorak and Grbić-Galić 1991; Kropp *et al.* 1994a).

1.6.2 Dibenzothiophene ring cleavage

Fluorene and its heteroatomic analogs, dibenzothiophene, dibenzofuran, and carbazole (Figure 1.9), are components of creosote, crude oils and shale oils (Fowler *et al.* 1994; Mueller *et al.* 1989; Later *et al.* 1981). The aerobic biodegradation of each of these compounds has been observed and investigated to various extents. Fluorene has been studied as a model polycyclic aromatic hydrocarbon (Grifoll *et al.* 1992; Trenz *et al.* 1994; Casellas *et al.* 1997). Dibenzothiophene serves as the archetypal condensed thiophene. Research has been done to predict the fate of dibenzothiophene and its homologues in petroleum-contaminated environments (Atlas *et al.* 1981; Fedorak and Westlake 1984a; Hosteller and Kvenvolden 1994) and much work has focussed on microbial processes to selectively remove the sulfur from dibenzothiophene (Krawiec 1990; Denome *et al.* 1994; Shennan 1996; Oldfield *et al.* 1997, 1998), the so-called biodesulfurization process. Similarly, laboratory investigations have been done to determine the fate of carbazole in the environment (Fedorak and Westlake 1984b, Gieg *et al.* 1996), with a lesser amount of effort into the selective removal of the nitrogen atom leading to biodenitrogenation (Benedik *et al.* 1998).

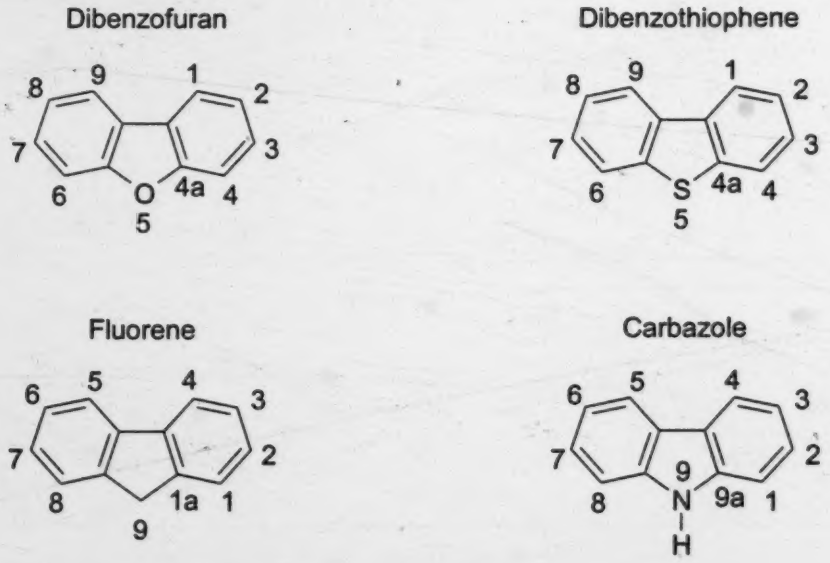


Figure 1.9 Fluorene, its analogs and their numbering conventions.

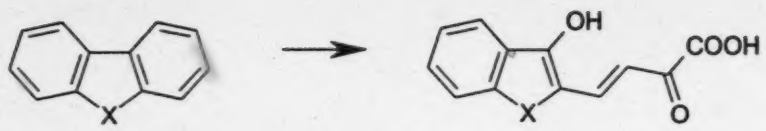
Dibenzothiophene biodegradation has been reviewed (Bressler *et al.* 1998; Kropp and Fedorak 1998), and recently carbazole metabolism, with an emphasis on biodenitrogenation, has been reviewed (Benedik *et al.* 1998). A more recent paper by Bressler and Fedorak (2000) compared and contrasted information on the aerobic bacterial metabolism of the four analogs shown in Figure 1.9.

In general, there are three modes of aerobic attack on dibenzothiophene and its analogs. One mode involves the dioxygenase attack at carbons 1 and 2 in dibenzothiophene and dibenzofuran or at carbons 3 and 4 of fluorene and carbazole (Figure 1.9 shows numbering conventions). This is analogous to naphthalene degradation which involves dioxygenation followed by *meta* cleavage (Grifoll *et al.* 1995), and I refer to this as the naphthalene-like attack. The second mode involves the initial oxidation of the methylene bridge in fluorene (Figure 1.9, carbon 9) or the sulfur atom of dibenzothiophene. I refer to this as the five-membered ring attack. The third mode is the so-called angular dioxygenase attack, which involves the oxidation of carbons 4a and 4 in dibenzofuran and dibenzothiophene (Figure 1.9), carbons 9a and 1 in carbazole (Figure 1.9), or carbons 1a and 1 in fluorene (Figure 1.9). These three modes of attack are discussed separately.

1.6.2.1 Dibenzothiophene naphthalene-like attack

Figure 1.10 illustrates that the four analogs of dibenzothiophene can all undergo naphthalene-like attack with a subsequent *meta*-ring cleavage yielding substituted 2-oxo-3-butenoic acids. Of the four compounds, this mode of attack of dibenzothiophene has been the most extensively reviewed (Foght *et al.* 1990; Gray *et al.* 1996; Shennan 1996; Kropp and Fedorak 1998).

The transformations of dibenzothiophene via the Kodama pathway by *Pseudomonas jianii* were shown to be cometabolic, because other substrates were required for growth and dibenzothiophene oxidation (Kodama 1977). Kodama *et al.* (1973) identified 4-(3'-hydroxy-2'-benzo[*b*]thienyl-2-oxo-butenoic acid (Figure 1.10) as a metabolite from dibenzothiophene. Saftić *et al.* (1993) and Kropp *et al.* (1997a) observed metabolites from methylated dibenzothiophenes indicating that these substituted compounds also undergo naphthalene-like attack.



X=S (Kodama et al. 1973)
Product = 4-(3'-hydroxy-2'-benzo[*b*]thienyl)-2-oxo-3-butenoic acid

X=O (Grifoll et al. 1995b)
Product = 4-(3'-hydroxy-2'-benzo[*b*]furanyl)-2-oxo-3-butenoic acid

X=NH (Grifoll et al. 1995b)
Product = 4-(3'-hydroxy-2'-indolyl)-2-oxo-3-butenoic acid

X=CH_2 (Grifoll et al. 1995b; Casellas et al. 1997)
The formation of 4-(3'-hydroxy-2'-indenyl)-2-oxo-3-butenoic acid was postulated but not detected.

Figure 1.10 *meta*-Cleavage products after dioxygenase attack at carbons 1 and 2 in dibenzofuran and dibenzothiophene, and carbons 3 and 4 in carbazole and fluorene.

The initial oxygenation steps of the Kodama pathway were elucidated by Laborde and Gibson (1977) who used succinate-grown cultures of a *Beijerinckia* sp. to cometabolize dibenzothiophene. A metabolite, (+)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene, accumulated transiently in the culture. This compound was purified and was converted to 1,2-dihydroxydibenzothiophene by crude cell extracts and purified 1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase (Laborde and Gibson 1977). Resnik and Gibson (1996) demonstrated that the naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4 produces the (+)-1*R*,2*S*-dihydrodiol from dibenzothiophene.

Denome *et al.* (1993) cloned and sequenced a 9.8-kb DNA fragment from *Pseudomonas* strain C18 that encodes the genes for dibenzothiophene biodegradation. The nine open reading frames in this fragment were designated *dox*ABDEFGHIJ. Three of the open reading frames, *dox*ABD, were identical to the *Pseudomonas putida ndo*ABC genes that encode naphthalene dioxygenase. DoxG was very similar to NahC (1,2-

dihydroxynaphthalene dioxygenase) from *P. putida* at both the nucleotide and predicted amino acid levels. Strain C18 grew on naphthalene as its sole carbon source, and Denome *et al.* (1993) showed that a single genetic pathway controls the metabolism of naphthalene to salicylaldehyde and the metabolism of dibenzothiophene to 3-hydroxy-2-formylbenzothiophene.

There are few reports of bacteria that can grow on dibenzothiophene via the Kodama pathway (Kropp *et al.* 1997b; Frassinetti *et al.* 1998). This pathway yields 3-hydroxy-2-formylbenzothiophene (which appears to accumulate in the growth medium) and presumably pyruvate which could be used for growth. There have been no specific investigations assessing the enzymes involved in the Kodama pathway. However, many bacteria can cometabolize dibenzothiophene, and these organisms can often use a range of aromatic hydrocarbons as carbons sources including naphthalene (Monticello *et al.* 1985; Saftić *et al.* 1993; Denome *et al.* 1993) and methylnaphthalenes (Saftić *et al.* 1993; Kropp *et al.* 1997b). Thus, there may not be a separate set of genes or enzymes for dibenzothiophene degradation via the Kodama pathway. The similarity of many of the *dox* genes with the *ndo* genes (Denome *et al.* 1993) and the ability of the naphthalene dioxygenase from strain NCIB 9816-4 to oxidize dibenzothiophene (Resnik and Gibson 1996) suggests that dibenzothiophene may simply serve as an alternate substrate for the naphthalene-degrading enzymes.

1.6.2.2 Dibenzothiophene five-membered ring attack

The sulfur atom in dibenzothiophene and the benzylic methylenic group in fluorene undergo bacterial oxidations. These initial oxidations seem to be essential for subsequent angular dioxygenase attack (to be discussed later).

The sulfur atom in dibenzothiophene is oxidized to the sulfoxide then to the sulfone in the 4S pathway (Krawiec 1990; Gallagher *et al.* 1993; Shennan 1996). Subsequently the thiophene ring is cleaved, leaving 2-hydroxybiphenyl, and releasing sulfite which spontaneously oxidizes to sulfate. Bacteria such as *Rhodococcus* sp. strain IGTS8 (Kilbane and Jackowski 1992), *Corynebacterium* sp. [later reclassified as *Rhodococcus* (Omori *et al.* 1995)] strain SY-1 (Omori *et al.* 1992), and *Rhodococcus erythropolis* strain D-1 (Izumi *et al.* 1994), are capable of this mode of attack, and they

use dibenzothiophene as their sole sulfur source. Because the carbon skeleton of the compound is unaltered, the 4S pathway is deemed the most attractive for a commercial biodesulfurization process (Rhodes 1995). Thus, the genetics and enzymology of the 4S pathway have received much attention (Denome *et al.* 1994; Gray *et al.* 1996; Piddington *et al.* 1995; Oldfield *et al.* 1997, 1998).

The genes responsible for dibenzothiophene desulfurization are located in a single operon on a circular plasmid in *Rhodococcus* strain IGTS8 (Oldfield *et al.* 1998). The gene cluster responsible for desulfurization has been cloned and sequenced (Denome *et al.* 1993; 1994; Piddington *et al.* 1995) and the promoter and regulatory regions have been studied (Li *et al.* 1996). The cluster contains three open reading frames designated *dszA*, *dszB*, and *dszC* (Gray *et al.* 1996) (called *soxA*, *soxB*, and *soxC* by Denome *et al.* 1994). All three enzymes have been purified and their activities studied (Gray *et al.* 1996).

A monooxygenase that oxidizes dibenzothiophene to the sulfoxide and then to the sulfone in a two-step process is encoded by the *dszC* gene. The first oxidation step is about one-tenth the rate of the second step (Gray *et al.* 1996). This enzyme appears to be specific for sulfoxidation because no other oxidized products of dibenzothiophene were detected. Lei and Tu (1996) over expressed the *dszC* gene in *Escherichia coli* and purified the enzyme which is a homodimer of 90,200 Da. Using $^{18}O_2$, they confirmed that both oxygen atoms in the sulfone were derived from molecular oxygen. The product of the *dszA* gene is another monooxygenase which oxidizes the sulfone to 2-(2'-hydroxyphenyl)benzenesulfinate (2'-hydroxybiphenyl-2-sulfinic acid, in Figure 1.11). The two monooxygenases require NADH and FMN for their catalytic activities. The product of the *dszB* gene is a novel desulfinase that converts 2-(2'-hydroxyphenyl)benzenesulfinate to 2-hydroxybiphenyl with the release of sulfite (Gray *et al.* 1996). This desulfinase controls the rate of the desulfurization of dibenzothiophene in strain IGTS8 because it is the slowest of the three enzymes.

All three enzymes (the monooxygenase that oxidizes dibenzothiophene to the sulfone, the monooxygenase that oxidizes the sulfone, and the desulfinase) are colorless, indicating that they contain no tightly associated chromophores (Gray *et al.* 1996). None of the enzymes were inhibited by the addition of EDTA, indicating that they did not

require metal ions as cofactors. The addition of Fe^{3+}, Fe^{2+} or Cu^{+} to reaction mixtures did not enhance the rates of the reactions of the monooxygenases (Gray *et al.* 1996).

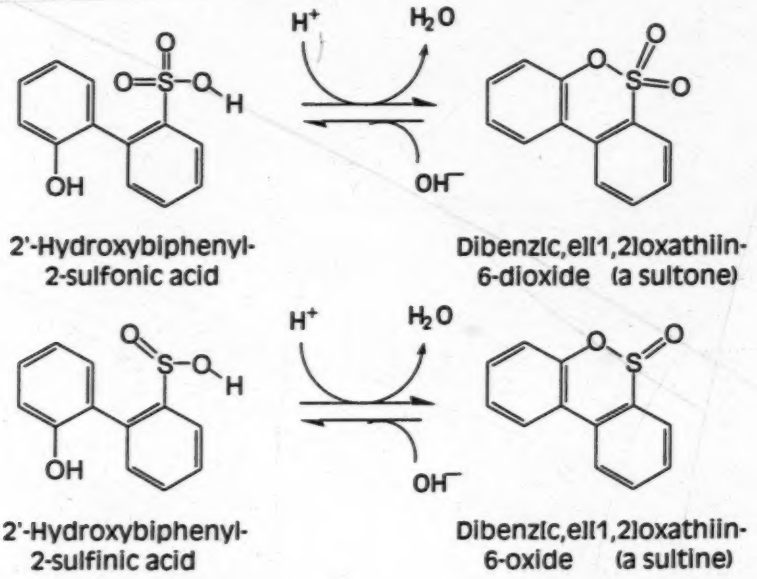


Figure 1.11 Acid catalyzed cyclization of 2'-hydroxybiphenyl-2-sulfonic acid and 2'-hydroxybiphenyl-2-sulfinic acid to form the sultone and sultine, respectively (after Olsen *et al.* 1993).

Other enzymes and bacteria are able to oxidize the sulfur atom in dibenzothiophene. For example, the naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4 oxidizes the sulfur atom, yielding the sulfoxide but not the sulfone (Resnik and Gibson 1996). Similarly, a carbazole 1,9a-dioxygenase oxidizes dibenzothiophene to its sulfoxide (Nojiri *et al.* 1999). Dibenzothiophene oxidation to its sulfone, which accumulated as an apparent dead end product, has been reported by several investigators working with different bacteria (Kodama *et al.* 1970; Labord and Gibson 1977; Mormile and Atlas 1988, Kropp *et al.* 1997b). In contrast, van Afferden *et al.* (1990, 1993) described *Brevibacterium* sp. strain DO that initially oxidizes the sulfur atom to the sulfoxide and then the sulfone, and subsequently oxidizes dibenzothiophene sulfone to carbon dioxide and sulfate. This metabolic pathway is described in more detail under the heading of "angular dioxygenase attack".

1.6.2.3 Dibenzothiophene angular dioxygenase attack

Dibenzofuran and carbazole are susceptible to angular dioxygenase attack (Bressler and Fedorak 2000), whereas this mode of attack has not been observed to occur directly on dibenzothiophene and fluorene. However, there are reports of angular attack on the latter two compounds after they have been initially oxidized to dibenzothiophene sulfone and 9-fluorenone, respectively. The angular attack on dibenzofuran has been studied more extensively than that on the other three compounds and was reviewed more extensively by Bressler and Fedorak (2000).

Dibenzothiophene biodegradation can occur via an angular dioxygenation, after the oxidation of the sulfur atom. van Afferden *et al.* (1990, 1993) described *Brevibacterium* sp. strain DO that initially oxidizes the sulfur atom to the sulfoxide and then the sulfone (Figure 1.12). Only after the sulfone has been formed does the angular attack occur (Figure 1.12). This pathway results in the complete mineralization of dibenzothiophene with the release of the sulfur atom as sulfite, which then oxidizes to sulfate. Because strain DO cometabolized fluoren-9-one (the carbon analogue of dibenzothiophene sulfoxide) to 1,1a-dihydrodihydroxyfluoren-9-one, van Afferden *et al.* (1993) proposed that dibenzothiophene sulfone was oxidized at the angular position in a similar manner as fluoren-9-one, yielding 4,4a-dihydroxy-4-hydrodibenzothiophene sulfone (shown in square brackets in Figure 1.12), which could not be isolated. This compound is an unstable hemimercaptal (S-oxidized form) and is expected to spontaneously decay to form 2',3'-dihydroxybiphenyl-2-sulfinic acid (van Afferden *et al.* 1993). Strong evidence for the formation of the sulfinated dihydroxybiphenyl was provided, but this metabolite could not be isolated. The mechanism for the cleavage of the C—S bond in a hemimercaptal (S-oxidized form) is analogous to the cleavage of the C—O bond in a hemiacetal (van Afferden *et al.* 1993), and the former mechanism is shown in Figure 1.13. Using a similar approach with dibenzothiophene and fluoren-9-one oxidation, Dahlberg *et al.* (1993) also suggested the angular oxidation of the former compound by an *Arthobacter* sp. resulting in C—S bond cleavage.

There are no detailed studies of the enzymology or molecular biology of the angular attack of dibenzothiophene sulfone described by van Afferden *et al.* (1990, 1993) or other investigators.

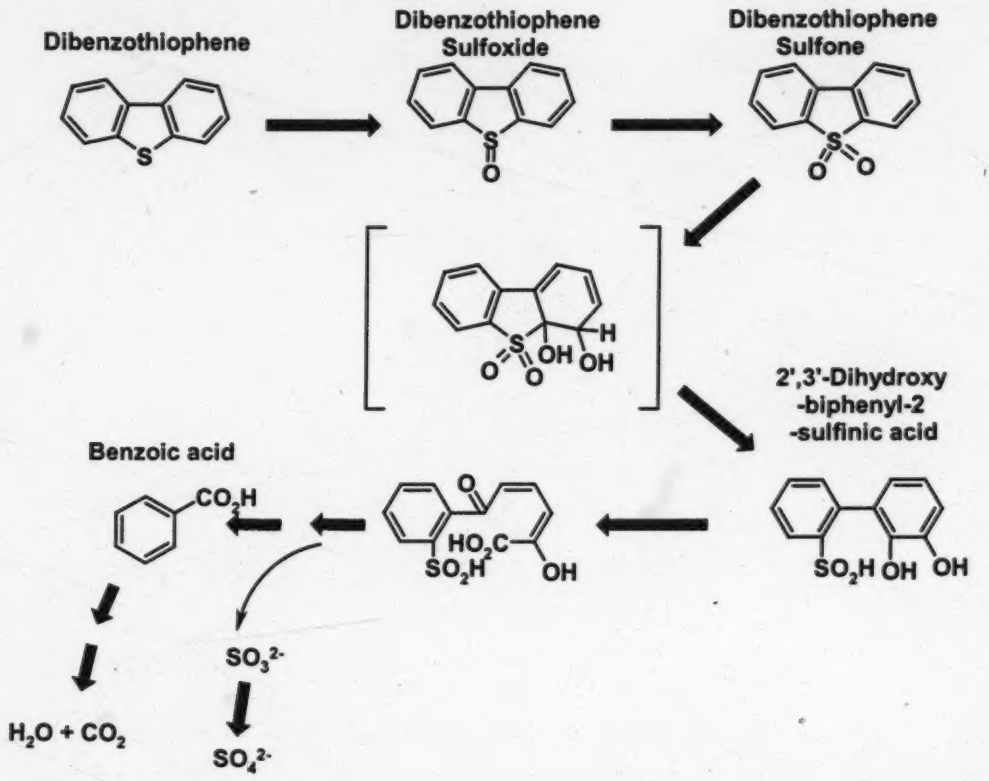


Figure 1.12 The pathway for dibenzothiophene metabolism by *Brevibacterium* sp. DO. The hypothesized product of angular dioxygenation is shown in brackets and this is the sulfoxidized hemimercaptal shown in Figure 1.13 (after van Afferden *et al.* 1993).

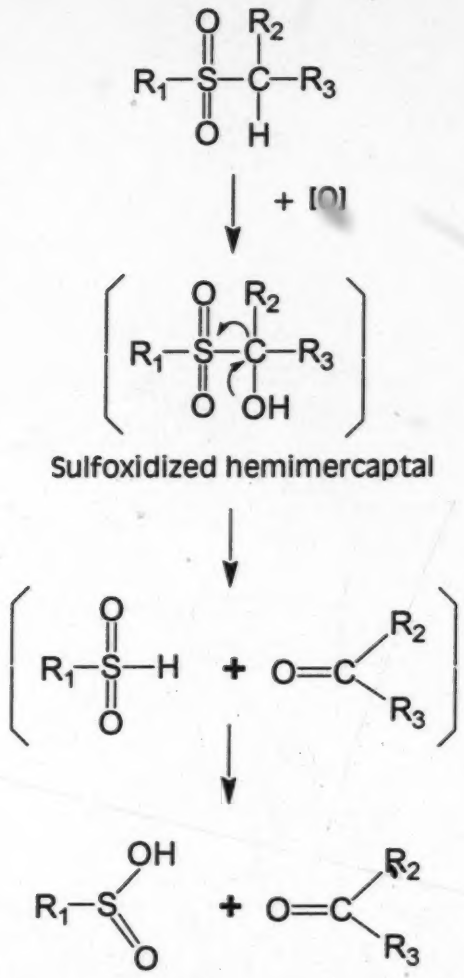


Figure 1.13 The C—S bond cleavage mechanism for a sulfoxidized hemimercaptal (after van Afferden *et al.* 1993).

1.7 CHALLENGES OF RING CLEAVAGE

A common feature of those sulfur heterocycle ring cleavages that have been studied in some detail is that the C–S bond is the most susceptible to cleavage. The specificity of this reaction is illustrated by the modified 4S pathway (Figure 1.8). The metal-catalyzed hydrodesulfurization (Figure 1.14) also shows this selectivity.

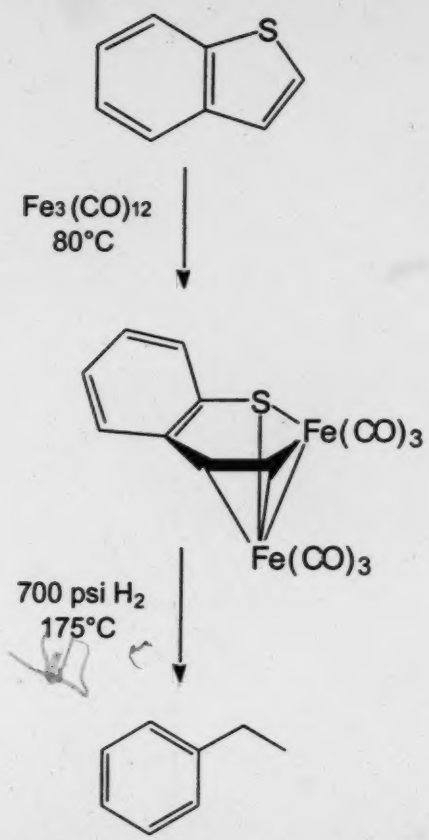


Figure 1.14 Example of insertion of a metal into the C–S bond of benzothiophene and subsequent hydrodesulfurization. The major product is ethylbenzene and the sulfur is removed as iron sulfide (after Myers *et al.* 1995).

Sabbah (1979) calculated the strength of the C–S bonds in thiophene, benzothiophene and dibenzothiophene to be approximately 340 kJ/mol (Table 1.1). This value is slightly greater than the thiol bond in methylmercaptan (CH_3–SH) and the sulfide bond in dimethylsulfide (CH_3–SCH_3) (Table 1.1). Sabbah (1979) also calculated the average strength of the C–C bond in benzene to be 505 kJ/mol, and the bond strengths for the single and double carbon bonds are also shown in Table 1.1. Comparing the C–C bond strengths to the C–S bond strengths in thiophenes (Table 1.1) suggests that the heteroatomic bonds are the weakest in these molecules. Thus, one would predict the C–S bond would be the most susceptible to cleavage.

Table 1.1 Bond strengths in selected compounds

Bond	Bond strength (kJ/mol)	Reference
C–C bonds		
$H_3C–CH_3$	376	Lide (1995)
$H_2C=CH_2$	733	Lide (1995)
C–C in benzene	505	Sabbah (1979)
$H_3C–CH_2CH_3$	330	Vedeneyev *et al.* (1966)
$H_3C–COCH_3$	290	Vedeneyev *et al.* (1966)
C–S bonds		
C–S in thiophene	341	Sabbah (1979)
C–S in benzothiophene	339	Sabbah (1979)
C–S in dibenzothiophene	338	Sabbah (1979)
$HS–CH_3$	312	Lide (1995)
$H_3C–SCH_3$	308	Lide (1995)
$H_3C–SO_2CH_3$	280	Lide (1995)
$H_3C–SCH_2C_6H_5$	257	Lide (1995)
$H_3C–SO_2CH_2C_6H_5$	221	Lide (1995)
H–C bonds		
$H–CH_3$	438	Lide (1995)
$H–CH_2OH$	410	Lide (1995)
H–CHO	364	Lide (1995)

Another common feature of the aerobic microbial attack of sulfur heterocycles is the addition of one or more oxygen atoms to the molecule (Figures 1.7, 1.8, 1.12, 1.13). With the exception of the Kodama pathway, oxygen is typically added to the sulfur atom and/or to the adjacent carbon atom. Second to fluorine, oxygen has the next highest electronegativity, and the presence of oxygen can weaken nearby bonds. Some examples of this phenomenon are given in Table 1.1 The strength of the H–C bond in methane is 438 kJ/mol. The addition of an OH group weakens this bond strength to 410 kJ/mol in methanol. Further oxidation to formaldehyde decreases the H–C bond strength to 364 kJ/mol. C–C bonds can also be weakened be the addition of oxygen. The C–C bond strength in propane (330 kJ/mol) is decreased to 290 kJ/mole in acetone (Table 1.1). In addition, the oxidation of the sulfur atom in dimethyl sulfide to give dimethyl sulfone

decreases the C–S bond strength from 308 to 280 kJ/mol. Similarly, oxidation of methyl tolyl sulfide to the corresponding sulfone decreases the C–S bond strength from 257 to 221 kJ/mol. Given that the C–S in a sulfur heterocycle is likely the weakest bond in the molecule, the addition of oxygen to the sulfur atom or to a carbon atom adjacent to the sulfur atom, would further weaken the C–S bond, thereby increasing the possibility of ring cleavage between these two atoms. Figure 1.13 illustrates the spontaneous cleavage of the C–S bond in a sulfoxidized hemimercaptal, which is the mechanism proposed by van Afferden *et al.* (1993) leading to the thiophene ring cleavage by their bacterial isolate. Also, the addition of an oxygen atom to a carbon atom adjacent to a sulfur atom facilitates cleavage by thioester hydrolysis.

1.8 RESEARCH OVERVIEW AND OBJECTIVES

The research done during this project focussed on the possible fates of known condensed thiophene metabolites. Benzothiophene sulfones, benzothiophene-2,3-diones, and 3-hydroxy-2-formylbenzothiophene are all previously identified products of dibenzothiophene and benzothiophene biodegradation. At the commencement of this investigation, nothing was known about the fates of these identified metabolites. The goal of this project was to understand possible pathways involved in the biodegradation of these metabolites in the environment. Strict biodesulfurization was not one of the goals of this investigation. In order to truly understand the biodegradation pathways involved in the biodegradation of condensed thiophenes it is imperative that all possible metabolites be identified.

Generally most condensed thiophene biodegradation studies, and most biodegradation studies in general, tend to demonstrate oxidation and a subsequent series of reactions leading to smaller products. This dissertation shows that the formation of significantly larger and more complex products occurs through condensation and oxidation reactions (chapters 4, 5 and 6). These types of condensation metabolites are not novel, as evidenced by Kropp *et al.* (1994b) but are often overlooked nonetheless. Identification of larger products is often not achieved or simply ignored due to the increasing difficulty of identifying these compounds. These larger compounds are often not amenable to routine analytical procedures such as gas chromatography or high

performance liquid chromatography when conducted under "standard" conditions. As demonstrated in this dissertation, identification of these compounds often requires additional procedures such as derivatization and more complicated handling procedures during sample preparation. The identification of all possible degradation products is required to generate accurate environmental impact assessments and toxicological assessments for biodegradation processes.

At the outset of this study it was decided to undertake a three pronged focus during investigations. Three known condensed thiophene metabolites were chosen for parallel biodegradation studies. Studies of the diones and 3-hydroxy-2-formylbenzothiophene were hampered by instability and abiotic chemical reactions. It was therefore often necessary to devote large amounts of effort into characterizing abiotic properties of these metabolites in addition to biodegradative processes. The specific objectives of the research in this dissertation were as follows:

Chapter 2. To isolate and identify a bacterial culture capable of degrading benzothiophene sulfone and methylbenzothiophene sulfones as sole carbon, energy and sulfur sources and demonstrate mineralization and sulfur release as sulfate.

Chapter 3. To try to further characterize sulfone biodegradation by investigating the effects of using extremely heavy inocula and the presence of 5-fluorobenzothiophene sulfone on a culture able to degrade benzothiophene sulfone.

Chaper 4. To produce enough purified 3-hydroxy-2-formylbenzothiophene to investigate its abiotic chemical properties, prior to conducting studies of its biodegradation.

Chapter 5. To investigate the abiotic chemical properties of benzothiophene-2,3-dione and the susceptibility of it or its abiotic reaction products to biodegradation.

Appendix A Purified laccase from the white rot fungus *Coriolopsis gallica* was tested for its ability to oxidize the polycyclic aromatic hydrocarbon fluorene and five related polycyclic heterocycles.

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2. BIODEGRADATION OF BENZOTHIOPHENE SULFONES BY A FILAMENTOUS BACTERIUM*

2.1 INTRODUCTION

Numerous condensed thiophenes are found in petroleum (Czogalla and Boberg 1983). Among these are the benzothiophenes and dibenzothiophenes which have been shown to be removed from petroleum by aerobic microbial activities in laboratory experiments (Fedorak and Westlake 1983; Bayona *et al.* 1986; Foght and Westlake 1988) and in oil-contaminated environments (Atlas *et al.* 1981; Hostettler and Kvenvolden 1994). However, the fate of these condensed thiophenes or their metabolites in the environment has not been determined.

Laboratory investigations with pure condensed thiophenes have identified many oxidized metabolites in aerobic cultures (see review by Kropp and Fedorak 1998), providing clues on how biodegradation might occur in the environment. The most extensively studied sulfur heterocycle is dibenzothiophene, and its most commonly identified metabolites are 2-hydroxy-3-formylbenzothiophene, dibenzothiophene sulfoxide, and dibenzothiophene sulfone (Kodama *et al.* 1970, 1973; Laborde and Gibson 1977; Monticello *et al.* 1985; Foght and Westlake 1988; Mormile and Atlas 1988; Crawford and Gupta 1990).

Only one study has attempted to detect further biodegradation of these metabolites. Mormile and Atlas (1988) used mixed cultures inoculated with soil and with sediment of a polluted creek and observed that 2-hydroxy-3-formylbenzothiophene was depleted from both of the mixed cultures relative to the sterile controls. As well, CO_2 production from the sulfoxide and sulfone was observed in the mixed culture inoculated with sediment. However, the biodegradation of these metabolites did not lead to release of sulfate into the medium. Thus, Mormile and Atlas (1988) concluded that while these metabolites were further degraded, with the release of CO_2, they were not completely mineralized.

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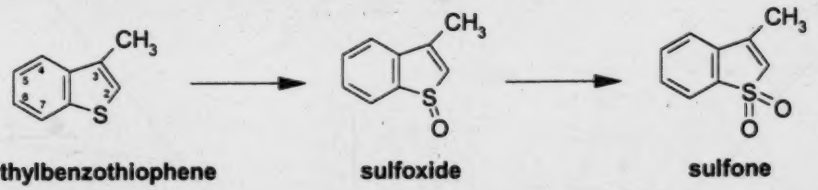
Sandhya *et al.* (1995) reported that some *Nocardioides* strains can use benzothiophene as their sole source of carbon and energy. However, most other reports indicate that benzothiophenes cannot serve as sole carbon and energy sources for microorganisms, but these condensed thiophenes can be cometabolized (Sagardía *et al.* 1975; Bohonos *et al.* 1977; Fedorak and Grbic′-Galic′ 1991; Kropp *et al.* 1994). The microbial oxidation of several benzothiophenes leads to the formation of sulfones. For example, Fedorak and Grbic′-Galic′ (1991) and Selifonov *et al.* (1996) identified the sulfone as a product from 3-methylbenzothiophene oxidation. Figure 2.1 shows the sequential oxidation of 3-methylbenzothiophene to the sulfone as proposed by Fedorak and Grbic′-Galic′ (1991). Kropp *et al.* (1994) detected sulfones from benzothiophene and all six of the methylbenzothiophenes that were studied in aerobic bacterial cultures growing on 1-methylnaphthalene. Similarly, Kropp *et al.* (1996) detected sulfones from three of the six dimethylbenzothiophenes that were studied.



3-Methylbenzothiophene **sulfoxide** **sulfone**

Figure 2.1 The sequential oxidation of 3-methylbenzothiophene to its sulfone (after Fedorak and Grbic′-Galic′ 1991).

The objective of this investigation was to determine whether some of these sulfones were susceptible to biodegradation. Because of their prevalence as microbial metabolites and their ease of synthesis, the sulfones of benzothiophene, 3-methylbenzothiophene and 5-methylbenzothiophene were used in the studies described in this paper. Initial experiments demonstrated that benzothiophene sulfone could be biodegraded by a mixed culture of petroleum-degrading bacteria. Subsequently, a filamentous bacterium, designated strain DB1, was isolated from one of the mixed cultures and this organism was identified as a member of the genus *Pseudonocardia* by 16S rRNA gene sequencing. The isolate could grow using the three sulfones as its sole source of carbon, sulfur and energy. Sulfate and sulfite were detected in the culture medium when the sulfones were consumed, providing evidence of thiophene ring cleavage (Bressler *et al.* 1998).

2.2 MATERIAL AND METHODS

2.2.1 Chemicals

Benzothiophene (99%) was purchased from Aldrich (Milwaukee, WI). 3-Methylbenzothiophene (98%) and 5-methylbenzothiophene (98%) were bought from Lancaster Synthesis (Windham, NH). These were used to synthesize the corresponding sulfones by the method of Bordwell *et al.* (1949), giving benzothiophene sulfone (mp 142-143°C), 3-methylbenzothiophene sulfone (mp 144-145°C) and 5-methylbenzothiophene sulfone (mp 121-122°C). Gas chromatography (GC) analyses showed that the sulfones were greater than 97% pure.

Sodium benzoate, *n*-hexadecane, and naphthalene were purchased from Fisher Scientific Company (Fair Lawn, NJ). D-Glucose was purchased from BDH Inc. (Toronto, ON). Dibenzothiophene sulfone (97%), hexadecanoic acid (99%), and 2-sulfobenzoic acid were obtained from Aldrich. Dibenzothiophene (>98%) was purchased from Fluka (Buch, Switzerland). Indole and phenol were obtained from Matheson, Coleman, & Bell (Norwood, OH) and Mallinckrodt (St. Louis, MO), respectively. Dibenzothiophene sulfoxide was purchased from ICN Pharmaceuticals, Inc (Plainview, NY).

2.2.2 Media

Mineral salts medium was used for all liquid cultures. The first mineral medium used, designated B+NP, was described by Fedorak and Westlake (1984). Each litre of medium contained 1 mL of trace metals solution (Fedorak and Grbic´-Galic´ 1991).

Sulfate-free medium was used to determine whether the sulfones could serve as a sole sulfur source. Water was purified by passing distilled water through a Milli-Q purification system (Continental Water Systems, El Paso, TX). Sulfate was removed from glassware by soaking glassware in 4 M HCl for 30 min and rinsing with Milli-Q water. The composition of the sulfate-free medium was the same as for B+NP medium except the chloride salts, NaCl, $FeCl_2 \cdot 4H_2O$, and $MgCl_2$ were substituted for equal masses of the sulfate salts Na_2SO_4, $FeSO_4 \cdot 7H_2O$, and $MgSO_4 \cdot 7H_2O$.

Plate Count Agar and International Streptomyces Project-3 (ISP-3) medium, both purchased from Difco Laboratories (Detroit, MI), and maltose yeast extract medium

(MYM, Stuttard 1982) agar plates were used during the isolation stages of strain DB1. Cultures prepared for electron microscopy were incubated at 28°C on tomato-oatmeal agar which was prepared as described by Pridham *et al.* (1957) except that the pH was adjusted to pH 6.8 before sterilization and agar was added to 2.5% (w/v).

2.2.3 Biodegradation studies

For most experiments, shake-flask cultures consisted of 200 mL or 300 mL of one of the mineral salts media in a 500-mL Erlenmeyer flask. The inoculum was either a 5% (v/v) inoculum from another liquid culture or a suspension from a plate culture. The suspensions were made by adding 5 mL of the sterile liquid medium onto the plate and scraping colonies into the liquid. The sulfone concentrations ranged from 20 to 100 mg/L, and these were initially supplied as the sole carbon source, and later as the sole carbon and sulfur source. Shake-flask cultures were incubated in the dark on a rotary shaker at 250 rpm. All cultures were incubated at 28°C unless otherwise indicated.

In the first experiment to determine whether the sulfones of benzothiophene and 3-methylbenzothiophene were biodegradable, 200 mL of B+NP medium were supplemented separately with 1.5 mg of each of the sulfones. Two petroleum-degrading mixed cultures, ESSO AG and ERN BIO, (described by Fedorak *et al.* 1996) were used as inocula and the medium was supplemented with 200 µL of Prudhoe Bay crude oil. These viable cultures and the appropriate sterile controls were incubated for 21 days, then they were acidified with 2 mL of 2 M H_2SO_4 and extracted with 3 x 30-mL portions of dichloromethane. The extracts were combined, concentrated and dried over anhydrous sodium sulfate before GC analyses.

Two hundred milliliter shake-flask cultures were used to test for utilization of the sulfones. Originally, the sulfones were added to the flasks at concentrations of 20 mg/L, but this concentration was later increased to 100 mg/L in B+NP medium which contained nitrogen, phosphorus and sulfur sources.

2.2.4 Bacterial cultures

Strain DB1 was isolated from the ESSO AG mixed culture after several serial transfers in sulfate-free medium with benzothiophene sulfone as sole carbon and sulfur source. A pellet-forming, filamentous organism grew in these enrichment cultures. Pellets were taken from liquid enrichment cultures and streaked onto Plate Count Agar. Isolated colonies were subsequently repeatedly streaked on ISP-3 plates. This yielded a pure culture of the filamentous bacterium that was able to utilize the benzothiophene sulfones as sole carbon and energy source in liquid medium.

Pseudonocardia compacta ATCC 35407 was obtained from the American Type Culture Collection (Manassas, VA).

2.2.5 Temperature and pH optima

Strain DB1 cultures were incubated at a range of temperatures (4°C to 37°C) and at a range of pH (pH 5.5 to pH 9.5). The cultures were grown in 200 mL of sulfate-free medium with 100 mg/L benzothiophene sulfone in 500-mL Erlenmeyer flasks. Removal of benzothiophene sulfone was monitored by HPLC.

2.2.6 Release of inorganic sulfur

Percentage of sulfur released as sulfate was determined by incubating 1.2 L cultures of strain DB1 growing on 100 mg/L of the 5-methylbenzothiophene sulfone or 3-methylbenzothiophene sulfone as well as sterile controls in 2-L Erlenmeyer flasks that were capped with foam plugs. Fifty-milliliter samples were taken every 2 to 4 days, and after the pH was measured, the samples were frozen. At the conclusion of the experiment the samples were all thawed and filtered with a Whatman GF/C filter (pore size 0.7 μm, Fisher Scientific) to remove the filamentous biomass. The samples were then analyzed in triplicate for residual substrate and for sulfur release (see below).

2.2.7 Carbon and sulfur balances in sealed flasks

Carbon and sulfur balances were determined for the DB1 cultures grown on 100 mg/L of the various sulfones in sealed flasks. 300 mL of Sulfate-free medium in a sealed 500-mL sidearm Erlenmeyer flask was inoculated with a 5% (v/v) inoculum of strain DB1. All cultures and sterile controls were analyzed in triplicate. Ten milliliters of air were injected into the sealed flasks to supply a positive pressure and to supply additional oxygen. These triplicate cultures were incubated for 21 days and then sacrificed for biomass, dissolved organic carbon, CO_2 release, and sulfate release. Filamentous bacterial biomass were collected on Whatman GF/C filters (pore size 0.7 μm) which had been previously heated to 550°C to remove all traces of carbon. The filtered supernatant was then analyzed for inorganic sulfur species and for dissolved organic carbon. Three more replicate flasks were acidified with 5 mL of 5 M H_2SO_4 and then analyzed for CO_2.

2.2.8 Growth substrates

Strain DB1 was tested for its ability to utilize a wide range of non-volatile substrates as sole carbon and energy sources, including benzoate, benzofuran, dibenzothiophene, dibenzothiophene sulfone, dibenzothiophene sulfoxide, glucose, hexadecane, hexadecanoic acid, 2-sulfobenzoic acid, sulfolane (tetrahydrothiophene sulfone), thiophene-2-carboxylic acid, and the sulfones of benzothiophene, 3-methylbenzothiophene, 5-methylbenzothiophene. These compounds were added at 100 mg/L to 200 mL of B+NP medium in Erlenmeyer flasks with foam plugs. After inoculation with strain DB1, the cultures were incubated at 28°C with shaking. Some of the substrates tested were volatile (benzothiophene, 3-methylbenzothiophene, 5-methylbenzothiophene, indole, naphthalene, and phenol) and these were individually added to the wells in sealed well flasks. Strain DB1 was inoculated into B+NP medium and the vapors of the test substrate were allowed to contact the medium. Each culture was scored for pellet growth after a 4-week incubation period. Selected compounds which served as carbon and energy sources for strain DB1 were then tested with *P. compacta* (ATTC 35407) as carbon and energy sources for this closely related species.

2.2.9 Analytical methods

During the initial phase of this study when crude oil was used, the sulfones were analyzed by GC (Fedorak and Grbic'-Galic' 1991) using dibenzothiophene sulfone as an internal standard. Throughout the remainder of the study, culture supernatants were then analyzed by high performance liquid chromatography using a Hewlett Packard model 1050 chromatography system and a 125 mm x 4 mm (5 μm) LiChrospher 100 RP-18 column (Hewlett Packard) with a mobile phase of acetonitrile:water (40:60) and a flow rate of 1 mL/min. The effluent was monitored at 310 nm.

A method similar to that of Braun and Gibson (1984) was used to measure carbon dioxide production in the sealed cultures. The cultures were acidified with 5 mL of 5 M H_2SO_4 and pressurized slightly by injecting 10 mL of air into the 500-mL flasks containing the cultures. After equilibrating overnight, 0.5 mL samples of headspace gas were injected into a Hewlett Packard 5890 Series II GC equipped with a thermal conductivity detector and a 4 m x 0.3 cm O.D. stainless steel column packed with Poropak R (mesh size 80/100, Waters Associate Inc., Milford MA). The helium carrier gas flow rate was 23 mL/min. The temperatures of the oven, injector and detector were 35°C, 37°C and 80°C, respectively. A calibration curve was prepared by adding measured amounts of sodium bicarbonate to a series of sealed flasks containing 300 mL of uninoculated medium. The contents of these sealed flasks were acidified and treated in the same manner as the cultures prior to sampling for GC analyses.

Dissolved organic carbon was measured with an Ionics model 1505 carbon analyzer using combustion and a nondispursive infrared detector. Biomass carbon was determined by filtering the culture through Whatman GF/C glass microfibre filters. After drying under reduced pressure, the samples were analyzed with a Control Equipment Corporation 440 Elemental Analyzer.

Sulfate and sulfite determinations were performed using a Dionex Series 2000 i/sp ion chromatograph with an Ion Pac AS4A-SC 4-mm I.D. column and a conductivity detector. The presence of sulfite was verified by reacting a portion of the culture supernatant with sulfite oxidase (Sigma, St. Louis, MO) as outlined by Gray *et al.* (1996). After the enzymatic reaction was complete, ion chromatographic analysis of the reaction mixture showed no trace of sulfite.

2.2.10 Sequencing gene encoding the 16S rRNA

Chromosomal DNA from strain DB1 was isolated using Procedure 3 of Hopwood *et al.* (1998) with the modification that the strain DB1 mycelium was dispersed using a ground glass homogenizer before the DNA was extracted. Initial polymerase chain reaction (PCR) amplification of the gene encoding the 16S rRNA was performed using primers 110, and 111 (obtained from G.W. Stemke, University of Alberta) listed in Table 2.1 using the method described by Deng *et al.* (1992). Due to poor amplification by this method, PCR primers DBR1 and DBR2 were synthesized, based on limited sequence data obtained by using Primers 110 and 111 using an Applied Biosystems model 373A automated system (Department of Biological Sciences Sequencing Service, University of Alberta). Subsequently, primers DBR1 and DBR2 were used to amplify large quantities of a 1066 base pair fragment of the gene encoding the 16S rRNA. Primer DBR2 bound to a 5' region of the gene just downstream of primer 111 whereas DBR1 bound to the 3' region of the gene just downstream of primer 110 as shown in Figure 2.2. PCR amplification was performed with 1 μg of strain DB1 genomic DNA as template and 40 pmol of each primer in 100 μL reaction volumes. One unit of Expand™ Long Template PCR System (Boehringer Mannheim GmbH, Germany) was used in each reaction mixture which was denatured for 5 min at 95°C followed by 30 cycles at 95°C for 30 s, 60°C for 60 s, and 72°C for 90 s.

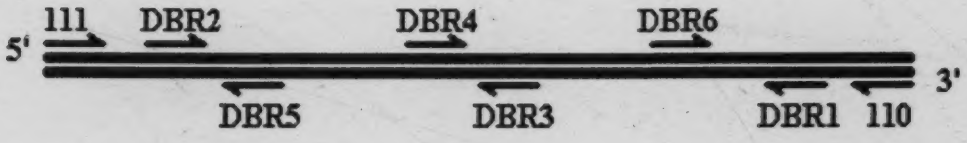


Figure 2.2 Sequencing strategy used to sequence isolate DB1 16S rRNA gene

Table 2.1. Primers used for the PCR amplification and for sequencing of strain DB1 16S rRNA.

Primer	Sequence
110	5' GGTAGGGATACCTTGTTACGACT 3'
111	5' AGAGTTTGATCCTGGCTCAGGA 3'
DBR1	5' CGTCATCCCCACCTTCCTCC 3'
DBR2	5' GGCGGACGGGTGAGTAACAC 3'
DBR3	5' CTCAGCGTCAGTATCGG 3'
DBR4	5' GGGTGCGAGCGTTGTCC 3'
DBR5	5' GGACAACGCTCGCACCC 3'
DBR6	5' CCGATACTGACGCTGAG 3'

Purification of PCR products was accomplished by the use of the band capture method (Zhen and Swank 1993). Primers DBR1 and DBR2 were then used to sequence the 5' and 3' regions of the gene as previously done with primers 110 and 111. From this sequence data, primers DBR3 through DBR6 were synthesized in order to completely sequence both strands of the gene as shown in Figure 2.2. All of the DBR series of primers were synthesized by the Department of Biological Sciences Synthesis Service, University of Alberta. The sequence obtained for the gene encoding the 16S rRNA was submitted, as accession number AF118130, to the Ribosomal Database Project for sequence comparison to known sequences (Maidak *et al.* 1997).

2.2.11 Scanning electron microscopy

Samples of strain DB1 were removed as agar plugs from cultures grown on tomato-oatmeal agar plates and these were prepared for scanning electron microscopy using a Emitek K1250 cryosystem. Samples were quick-frozen in liquid nitrogen slush and gold sputtered as described by Kernaghan *et al.* (1997). Scanning electron microscopy was performed using a Jeol JSM6301FXV.

2.3 RESULTS

In the presence of crude oil, mixed cultures degraded benzothiophene sulfone and 3-methylbenzothiophene sulfone. After 21 days of incubation, the ERN BIO mixed culture removed 18% of the benzothiophene sulfone and 36% of the 3-methylbenzothiophene sulfone whereas the ESSO AG mixed culture removed >99% of the benzothiophene sulfone and 95% of the 3-methylbenzothiophene sulfone. Thus, ESSO AG culture was used as an inoculum to attempt to isolate a pure culture of a benzothiophene sulfone-degrading bacterium. Strain DB1 was isolated and characterized, and its ability to mineralize the sulfones of benzothiophene, 3- and 5-methylbenzothiophene was studied.

2.3.1 Characteristics of strain DB1

Strain DB1 was isolated after repeated streaking and incubating on ISP-3 plates. It was found to be a filamentous Gram-positive bacterium that grew as pellets in liquid medium. When grown on tomato-oatmeal agar, strain DB1 produced an orange, unidentified pigment associated with the hyphae in the agar. When plate cultures of strain DB1 were left incubating for longer than 4 weeks, all aerial mycelium collapsed giving the colonies with a shiny yellow, flat appearance in contrast to the white, hairy, raised appearance of younger plate cultures.

Incubations of strain DB1 with 100 mg/L benzothiophene sulfone in sulfate-free medium at temperatures between 4°C and 37°C demonstrated that the isolate was able to utilize the sulfone at temperatures between 23 to 34°C. The optimum temperature for sulfone utilization was approximately 28 to 30°C. After 21 days of incubation, no utilization of the sulfone was observed at temperatures < 18°C or at 37°C.

Scanning electron microscopy suggested that strain DB1 produced aerial hyphae that later differentiated to produce chains of cylindrical spores. Figure 2.3a illustrates a 21-day-old colony grown on tomato oatmeal agar. Figure 2.3b illustrates typical aerial mycelia differentiated to yield chains of cylindrical spores. As shown in Fig. 2.3c and 2.3d, helically twisted hyphae, observed previously only in *P. compacta* (Henssen *et al.* 1983), as well as acropetal apical and intercalary swellings were observed.

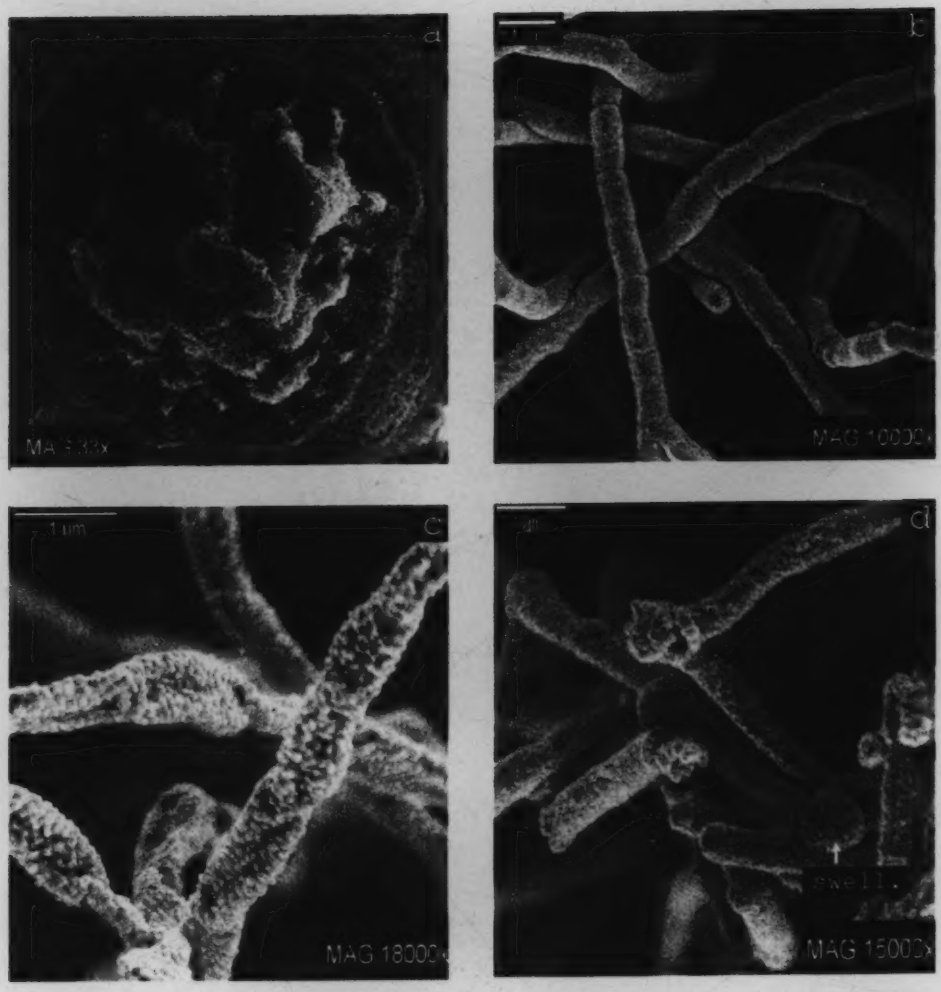


Figure 2.3 Morphology of a 21-day-old culture of strain DB1 observed by scanning electron microscopy. (a) A colony grown on tomato-oatmeal agar; (b) aerial mycelia showing chains of cylindrical spores; (c) examples of helically twisted hyphae; and (d) acropetal apical and intercalary swelling (swell.)

Based on the sequence comparisons of the gene encoding the 16S rRNA (Figure 2.4) and scanning electron microscopy, strain DB1 was grouped into the genus *Pseudonocardia*. Its production of an yellow-orange pigment and sporulation by

fragmentation is characteristic of many *Pseudonocardia* species (Henssen and Schäfer 1971). By sequence comparisons, the closest relative of strain DB1 was *Pseudonocardia halophobia* (Figure 2.4). A distinguishing characteristic of *P. halophobia* is its inability to grow on peptone, yeast extract agar with 3% NaCl (Akimov *et al.* 1989; Evtushenko *et al.* 1989). Strain DB1 was found to grow on this medium with 3% NaCl. Therefore, DB1 was not an isolate of *P. halophobia*.

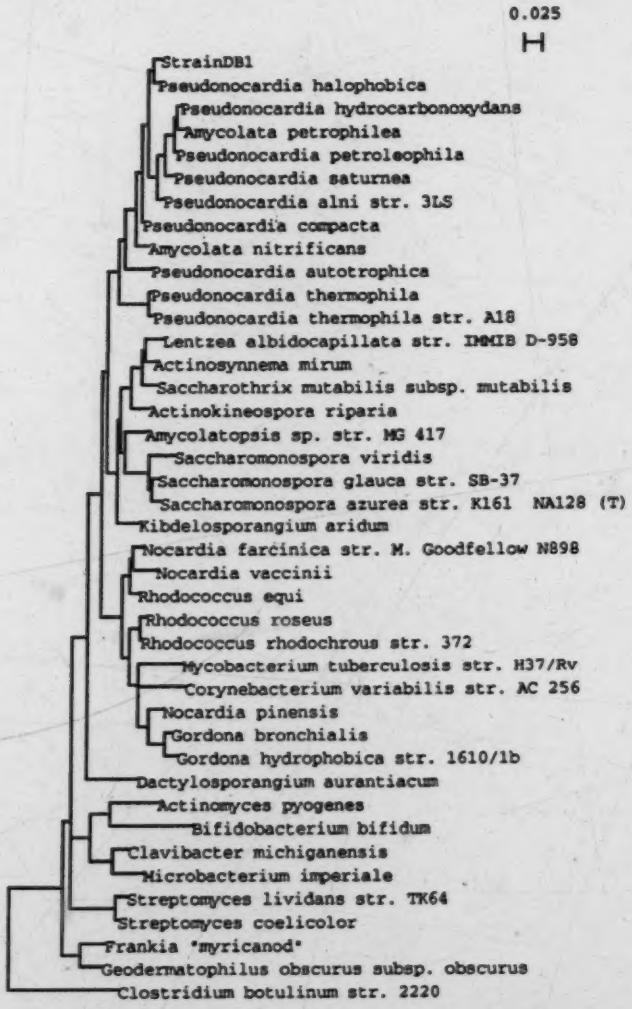


Figure 2.4 Dendrogram of bacteria with 16S rRNA gene sequences similar to that of strain DB1. Scale bar represents substitutions per site.

2.3.2 Growth substrates

Of the 20 compounds tested, only benzoate, glucose, and the sulfones of benzothiophene and 3- and 5-methylbenzothiophene supported good growth of isolate DB1 (Table 2.2). The isolate grew weakly on dibenzothiophene sulfone, and hexadecanoic acid. It did not grow on the hydrocarbons, nor on the heterocyclic analogs of benzothiophene that were tested (Table 2.2).

Because *P. compacta* appears to be a close relative of strain DB1 (Figure 2.4), *P. compacta* ATCC 35407 was tested on selected substrates that strain DB1 uses as sole carbon sources (Table 2.2). Although *P. compacta* could grow on benzoate and glucose, it did not grow on benzothiophene sulfone, 3-methylbenzothiophene sulfone or hexadecanoic acid which all serve as carbon and energy sources for strain DB1.

2.3.3 Acid production during growth

Equations [1] and [2] show the stoichiometry of complete mineralization of one mol of benzothiophene sulfone and methylbenzothiphene sulfone under aerobic conditions, respectively. They predict that the release of the sulfur atom as sulfate would yield two equivalents of protons.

$$C_8H_6O_2S + 10\,O_2 \rightarrow 2\,H_2O + 8\,CO_2 + 2\,H^+ + SO_4^= \quad \text{[equation 2.1]}$$

$$C_9H_8O_2S + 11.5\,O_2 \rightarrow 3\,H_2O + 9\,CO_2 + 2\,H^+ + SO_4^= \quad \text{[equation 2.2]}$$

An early observation with cultures of strain DB1 growing in sulfate-free medium with benzothiophene sulfone was a decrease in the pH of the medium which caused the cessation of sulfone biodegradation. Inoculating cultures of strain DB1 in sulfate-free medium with initial pH values between 5.5 and 9 demonstrated that benzothiophene sulfone degradation could occur under each of these conditions. However, when the pH of the medium fell to below pH 5, utilization of the sulfone ceased. Based on these observations, an initial pH of 7 was chosen for the preparation of sulfate-free medium for degradation studies.

Table 2.2. Alternate carbon sources tested for growth of strain DB1 and *P. compacta* ATCC 35407.

Compound	Growth[a] of Strain DB1	Growth[a] of *P. compacta*
Benzoate	+++	+++
Benzofuran	–	nt[b]
Benzothiophene	–	nt
3-Methylbenzothiophene	–	nt
5-Methylbenzothiophene	–	nt
Benzothiophene sulfone	++	–
3-Methylbenzothiophene sulfone	++	–
5-Methylbenzothiophene sulfone	++	nt
Dibenzothiophene	–	nt
Dibenzothiophene sulfone	+	–
Dibenzothiophene sulfoxide	–	nt
Glucose	+++	+++
Hexadecane	–	nt
Hexadecanoic acid	+	–
Indole	–	nt
Naphthalene	–	nt
Phenol	–	nt
2-Sulfobenzoic acid	–	nt
Sulfolane	–	nt
Thiophene-2-carboxylic acid	–	nt

[a] Relative amount of growth scored by examining the amount of pellet formation in cultures incubated for 4 weeks. "–" indicates no growth.
[b] nt, not tested

The initial culture pH affected the pellet size of strain DB1 in liquid culture. At high starting pH values, such as pH 8.5, strain DB1 tended to grow as a few larger pellets. As the starting pH was decreased towards pH 6, strain DB1 grew as more numerous smaller pellets.

2.3.4 Mineralization of the benzothiophene sulfones

Biodegradation of benzothiophene sulfone and 3-methylbenzothiophene sulfone was accompanied by a decrease in pH and an increase in sulfite and sulfate concentrations (Figures 2.5 and 2.6). The total molar quantities of these two anions accounted for nearly 50% of the sulfur in the benzothiophene sulfone (Figure 2.5). Similarly, Figure 2.6 shows that the consumption of 3-methylbenzothiophene sulfone by strain DB1 led to a release of sulfite and sulfate accounting for about 80% of the sulfur from the sulfone. In both experiments (Figures 2.5 and 2.6), the pH drop was observed after the sum of the sulfate and sulfite concentrations reached about 0.1 mM, indicating that the buffer capacity of the medium was exceeded by the corresponding release of H^+.

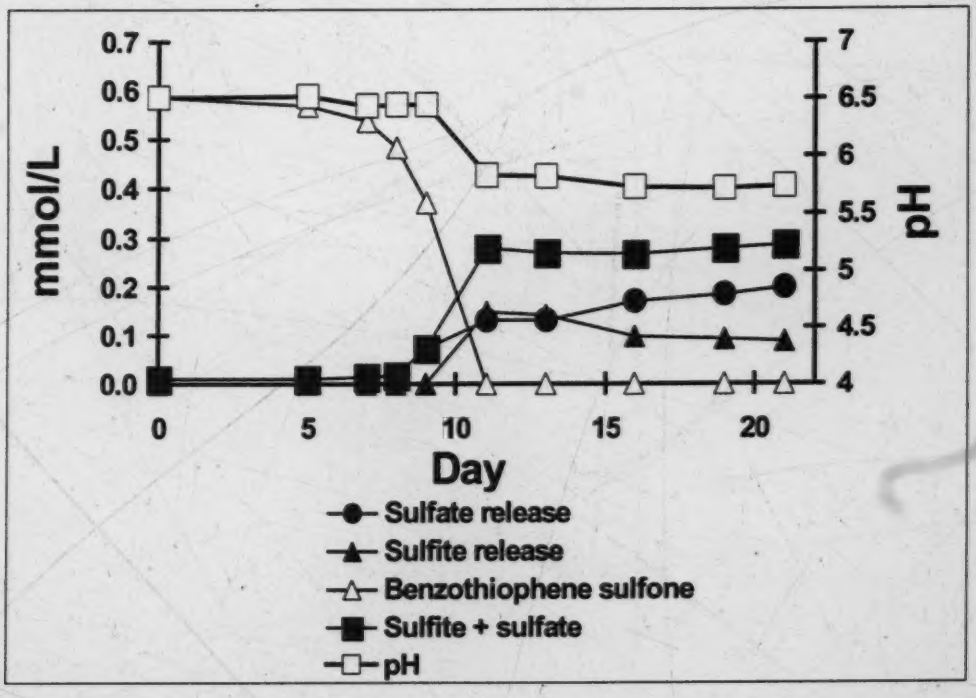


Figure 2.5 Changes in the medium during the growth of strain DB1 on benzothiophene sulfone. Error bars of one standard deviation were smaller than the size of the symbols used.

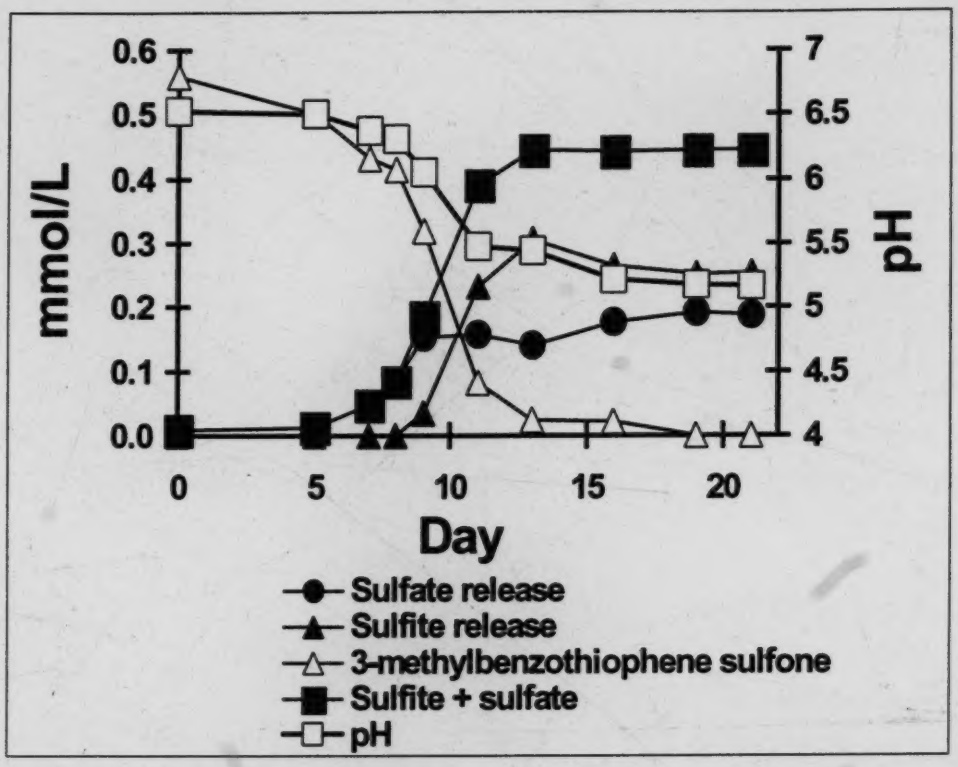


Figure 2.6 Changes in the medium during the growth of strain DB1 on 3-methylbenzothiophene sulfone. Error bars of one standard deviation were smaller than the size of the symbols used.

Cultures grown on benzothiophene sulfone and 3-methylbenzothiophene sulfone quickly consumed all their substrate releasing 57% and 62% of the carbon from these compounds as CO_2 (Table 2.3). In contrast, the cultures of strain DB1 incubated with 5-methylbenzothiophene sulfone consumed only 67% of the substrate over the 21-day period. CO_2 release accounted for 28% of the amount of carbon from the added 5-methylbenzothiophene sulfone (Table 2.3) which is equivalent to 43% of the consumed sulfone. The proportion of unidentified soluble organic carbon was 26% of the amount of substrate added to the cultures. This value was in the range of the proportions of soluble carbon found in the cultures containing the other two sulfones (35% and 17%, Table 2.3). The amount of carbon converted to biomass ranged from 15% to 19% of the amounts of

the three sulfones utilized by strain DB1. The overall recoveries of carbon ranged from 96% to 110%.

Sulfate and sulfite were found in the cultures grown on benzothiophene sulfone, and these accounted for 44% of the sulfur in the substrate (Table 2.3). Although sulfite was detected in an earlier experiment with 3-methylbenzothiophene sulfone (Figure 2.6), only sulfate was detected in the cultures grown on 3-methylbenzothiophene sulfone in the latter experiment (Table 2.3), and sulfate accounted for 77% of the sulfur in the consumed substrate. These values agree closely with the total amounts of inorganic sulfur shown released in Figures 2.5 and 2.6, where nearly 50% and 80%, respectively, of the organic carbon was converted to inorganic anions. The cultures grown on 5-methylbenzothiophene sulfone released 88% of the organic sulfur from the consumed substrate as sulfate (Table 2.3).

Table 2.3. Sulfate release and carbon balance for strain DB1 utilization of benzothiophene sulfone, 3-methylbenzothiophene sulfone and 5-methylbenzothiophene sulfone.

		SUBSTRATES		
		Benzothiophene sulfone	**3-Methylbenzo-thiophene sulfone**	**5-Methylbenzo-thiophene sulfone**
Sulfone Consumed (mmol)		0.16 ± 0.012	0.20 ± 0.008	0.10 ± 0.003
Percent of C found as	Residual substrate	0	0	33 ± 6
	CO_2	57 ± 4.5	62 ± 12	28 ± 1.9
	Unidentified organic C	35 ± 4.0	17 ± 2.9	26 ± 6.1
	Biomass C	19 ± 4.6	17 ± 3	15 ± 3.9
Total C recovered	(mmol)	1.4 ± 0.17	1.7 ± 0.33	1.4 ± 0.26
	(%)	110 ± 13	96 ± 19	102 ± 19
% Inorganic sulfur released		44 ± 5.5 a	77 ± 2.0 a	88 ± 16 b,c

a Sulfate and sulfite detected
b Only sulfate detected
c Percent based on sulfone consumed

2.4 DISCUSSION

Strain DB1 was found to belong to the genus *Pseudonocardia* based on sequence of the gene encoding the 16S rRNA and on physical morphology. Its ability to grow in the presence of 3% NaCl distinguished strain DB1 from *P. halophobia*. The substrate utililization pattern of strain DB1 was different from that of *P. compacta* ATTC 35407 (Table 2.2). Thus, isolate DB1 differs from its closest relatives shown in Figure 2.4.

Little is know about the mechanism of thiophene ring cleavage (reviewed by Bressler *et al.* 1998). Dibenzothiophene is the best studied compound, and two well documented cases show that it is oxidized to the sulfoxide and then to the sulfone prior to release of the sulfur atom. Gray *et al.* (1996) demonstrated that *Rhodococcus erythropolis* strain IGTS8 cleaves the thiophene ring of dibenzothiophene sulfone yielding 2-hydroxybiphenyl and sulfite. Strain IGTS8 is being studied extensively for its potential use in biodesulfurization (Gray *et al.* 1996). van Afferden *et al.* (1990, 1993) observed that *Brevibacterium* sp. DO also released sulfite from dibenzothiophene sulfone. However, their isolate continued to mineralize the hydrocarbon portion of the molecule to CO_2. Sulfite, which will be oxidized to sulfate, was found in some cultures of strain DB1, consistent with the findings of Gray *et al.* (1996) and van Afferden *et al.* (1990, 1993). This data indicates that the sulfur atom from the benzothiophene sulfones may also be released as sulfite.

Recently, Bambauer *et al.* (1998) reported that thiosulfate was detected as the end product of aerobic degradation of thiophene-2-carboxylic acid. However, no thiosulfate was detected in our ion chromatographic analyses of culture supernatants from strain DB1 grown on the sulfone of benzothiophene, 3- or 5-methylbenzothiophene.

The biodegradation of sulfolane (tetrahydrothiophene sulfone) has been reported (Chou and Swatloski 1983; Greene *et al.* 1998) and the near stoichiometric release of sulfate and a drop in the pH of the growth medium was observed by Chou and Swatloski (1983). This decrease in pH was used as the basis for a differential medium for the detection and enumeration of sulfolane-degrading bacteria (Greene and Fedorak 1998). As predicted by equation [2.1], the biodegradation of the benzothiophene sulfones resulted in the release of the sulfur atom as sulfate, as summarized in Table 2.3, and the

decrease in pH of the growth medium, as illustrated in Figures 2.5 and 2.6. However, strain DB1 will not grow on sulfolane.

In the cultures grown on 3-methylbenzothiophene sulfone, the pH dropped from 6.5 to 5.2 (Figure 2.6), whereas in the cultures that contained benzothiphene sulfone, the pH dropped from 6.5 to 5.8 (Figure 2.5). In each case, the sulfones were no longer detectable in these cultures after 21 days of incubation (Figures 2.5 and 2.6). Based on equations [2.1] and [2.2], a lower final pH (as a result of more H^+ being released from the sulfone) should be accompanied by a greater release of the sulfone sulfur as an inorganic form. Indeed this was observed, with about 80% of the sulfur from 3-methylbenzothiophene sulfone being detected as sulfate and sulfite (Figure 2.6) and only about 50% of the sulfur from benzothiophene sulfone being detected as these two anions (Figure 2.5). The lower amount of sulfur release from benzothiophene sulfone implies that more of the sulfur remained in an organic form in the cultures. Table 2.3 shows that 35% of the carbon from benzothiophene sulfone remained as unidentified soluble organic-C, whereas only 17% of the carbon from 3-methylbenzothiophene sulfone remained as unidentified soluble organic-C. These results are consistent with the hypothesis that more of the sulfur from benzothiophene sulfone remained as organic material.

Based on the data from the carbon balance experiment summarized in Table 2.3, 5-methylbenzothiophene sulfone was more difficult to attack than the 3-methyl isomer or benzothiophene sulfone. That is, over the 21-day incubation period, only one-third of the 5-methyl isomer was consumed whereas all of the other two sulfones were degraded. These results suggest that the position of the methyl group on the homocyclic ring hampers the microbial attack of the 5-methylbenzothiophene sulfone. The position of the alkyl group of methyldibenzothiophenes has been shown to influence the susceptibility of microbial attack; with the 2-methyl and 3-methyl isomers being degraded faster than the 4-methyl and 1-methyl isomers (Bayona *et al.* 1986).

To my knowledge, this is the first demonstration of the biodegradation of benzothiophene sulfone, and 3- and 5-methylbenzothiophene sulfones. The ability of *Pseudonocardia* DB1, to degrade these compounds demonstrates that these oxidized metabolites of condensed thiophenes could be subject to further biodegradation in contaminated environments. Although strain DB1 cannot oxidize the parent thiophenes to

sulfones, it would likely be able to grow in cooperation with another microorganism that produces these sulfones. For example, a recombinant bacterium containing naphthalene dioxygenase will catalyze monooxygenation reactions of numerous organosulfur compounds, including 3-methylbenzothiophene, to form sulfoxides and sulfones as dead-end products (Selifonov *et al.* 1996). Thus, sulfoxidation by aromatic hydrocarbon-degrading bacteria appears to be a fortuitous oxidation by the dioxygenase. This type of reaction could provide growth substrates for strain DB1, which has clearly been shown to mineralize the sulfones (Table 2.3). These interactions between microorganisms may play a key role in the removal of benzothiophenes from petroleum- or creosote-contaminated environments.

Although this study revealed that isolate strain DB1 has the ability to utilize and mineralize the sulfones of benzothiophenes, further investigations are needed to identify transient polar metabolites of these sulfones and to attempt to elucidate the pathway of the biodegradation of these compounds. This is the focus of ongoing studies in our laboratory. The data gathered from this work should help explain why the 5-methyl isomer is more difficult to degrade than 3-methylbenzothiophene sulfone.

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3. *PSEUDONOCARDIA* STRAIN DB1 UTILIZATION OF BENZOTHIOPHENE SULFONE AND SUBSTITUTED BENZOTHIOPHENE SULFONE

3.1 INTRODUCTION

Condensed thiophenes comprise a subgroup of the sulfur heterocycles and are found in crude oil, bitumen, and other forms of fossil fuels. It has been shown that sulfur is the third most abundant element in crude oil and condensed thiophenes contain a significant proportion of that sulfur (Speight 1980). These compounds consist of at least one five-member sulfur-containing thiophene ring and at least one conjugated aromatic benzene ring sharing two carbon atoms with the thiophene ring. They are known to be recalcitrant to biodegradation and among the most persistent contaminants in the environment (van Afferden *et al.* 1990). The fates of condensed thiophenes in the environment are not well understood but investigations into their biodegradation have been recently reviewed by Kropp and Fedorak (1998). There are two main problems with the presence of condensed thiophenes in these mixtures. First, upon combustion of these sulfur heterocycles, polluting and corrosive sulfur-containing emissions are released leading to the formation of acid rain. Second, some of these compounds have carcinogenic and toxic properties (Kropp and Fedorak 1998).

When contemplating bioremediation solutions to help eliminate the two above mentioned problems, it is beneficial to understand the possible metabolic pathways utilized by various microorganisms to degrade these types of compounds. Unfortunately, many of the catabolic pathways for condensed thiophenes remain unknown. Typically in order to elucidate a metabolic pathway, a pure chemical is used as a substrate to select for microorganisms capable of metabolizing or co-metabolizing the chemical as a carbon, energy, or even sulfur source. After a utilizing strain has been isolated, chemical analyses are used to identify accumulating or transient metabolites if they are present. These methodologies work well if metabolites are produced in quantities sufficient to allow significant accumulation.

Unfortunately, if the biodegradation is too efficient the process may have to be artificially inhibited in order to generate significant quantities of metabolites for

identification. One mechanism of achieving metabolite accumulation is the addition of a fluorinated analog which interferes with biodegradation by inhibiting enzyme activities or is poorly metabolized resulting in metabolite accumulation. It is known that the biodegradation of the fluorinated analogs of aromatic compounds as sole carbon, and energy sources is more restricted than that of the non-fluorinated analogs and that use of these fluorinated compounds is often the result of concurrent metabolism in the presence of suitable substrates (Neilson 2000). A second mechanism of enhancing metabolite production is by using concentrated washed cell suspensions and subjecting them to high concentrations of substrate (Casellas *et al.* 1998; Sabate *et al.* 1999; Mengs *et al.* 1999). In this situation, there may be a significant amount of biodegradation yielding intermediates.

Benzothiophene is a model condensed thiophene that has been subjected to microbial degradation experiments in order to determine its susceptibility to biodegradation (Sagardía *et al.* 1975; Bohonos *et al.* 1977; Fedorak and Grbić-Galić 1991; Kropp *et al.* 1994a). All reports in the literature reveal that benzothiophene cannot serve as a sole carbon and energy source and must be co-metabolized, with one exception (Sandhya *et al.* 1995). Two oxidized metabolites of benzothiophene reported in the literature are benzothiophene sulfone and 2-mercaptophenylglyoxalate (Kropp *et al.* 1994a; Eaton and Nitterauer 1994).

2-Mercaptophenylglyoxalate has been shown to exist as the cyclized compound benzothiophene-2,3-dione under acidic conditions (Eaton and Nitterauer 1994). Corresponding sulfones and diones have also been identified from biodegradation experiments with mono- and dimethylated benzothiophenes (Kropp *et al.* 1994a, 1996; Saftić *et al.* 1992; Selifonov *et al.* 1996). Fedorak and Grbić-Galić (1991) and Selifonov *et al.* (1996) identified the sulfone as a product from 3-methylbenzothiophene oxidation. There is only one published report of biodegradation of any of these metabolites (Bressler *et al.* 1999) and it is included in this dissertation as Chapter 2.

Generally, as biodegradation continues, the metabolites tend to become more oxidized and much more water soluble than the parent compounds which makes extraction into organic solvents for analysis much harder, if not impossible. Because of the increasing polarity of metabolites, it becomes increasingly difficult to separate and

identify them by conventional gas chromatography (GC) methods. Alternative techniques such as derivatization, the use of ion exchange resins, or high performance liquid chromatography (HPLC) must be incorporated. Thus, many earlier studies investigating the biodegradation of dibenzothiophene and benzothiophene identified initial metabolites such as 3-hydroxy-2-formylbenzothiophene (HFBT) and benzothiophene sulfone respectively, but complete pathways to mineralization were not elucidated. This is unfortunate because from an environmental standpoint, it is beneficial to understand which metabolites are produced and what effect they have on contaminated site characteristics, in terms of toxicity and carcinogenicity, when bioremediation is being considered as an option for remediation of contaminated environmental sites.

Another consequence of biodegradation is the possibility that the production of certain metabolites may result in abiotic chemical reactions including condensation reactions. These reactions must be identified and understood if the fate of all substrate carbon is to be accounted for and understood. Condensation reactions have been reported for benzothiophene sulfoxide by Kropp *et al.* (1994b) and for HFBT by Bressler and Fedorak (2001), included as Chapter 4 of this dissertation. The Diels-Alder type of condensation reported by Kropp *et al.* (1994b) (Figure 3.1) was reported to only occur with two molecules of benzothiophene sulfoxide but not with benzothiophene or benzothiophene sulfones. Condensation products (in general) are larger than the parent compounds and often have decreased solubility in the aqueous culture medium. Their large size, sometimes combined with the presence of polar functional groups, means that identification and quantitation through conventional solvent extraction and GC analysis may fail.

It is important to determine if the larger condensation products or the more polar metabolites of sulfur heterocycles have increased toxicities or carcinogenic properties. Zemanek (1994) found that the polar fraction from petroleum contaminated sites was the most toxic fraction. Belkin *et al.* (1994) found that when biodegradation of polycyclic aromatic hydrocarbons is incomplete, the potential exists for toxicity and genotoxicity enhancement. In contrast, Seymour *et al.* (1997) found that initial metabolites of some benzothiophenes and dibenzothiophene, namely benzothiophene sulfone, 3- and 5-methylbenzothiophene sulfones, benzothiophene-2,3-dione, 5- and 7-benzothiophene-2,3-

diones, dibenzothiophene sulfone and dibenzothiophene sulfoxide, were all less toxic than the parent condensed thiophene. If the further metabolites of these condensed thiophenes are more toxic than the parent compounds then bioremediation may be a less attractive treatment strategy. Little is known of the processes involved in the microbial breakdown and release of sulfur from these condensed thiophenes. Cleavage of the sulfur-carbon bonds in sulfur heterocycles is not well understood (Bressler *et al.* 1998).

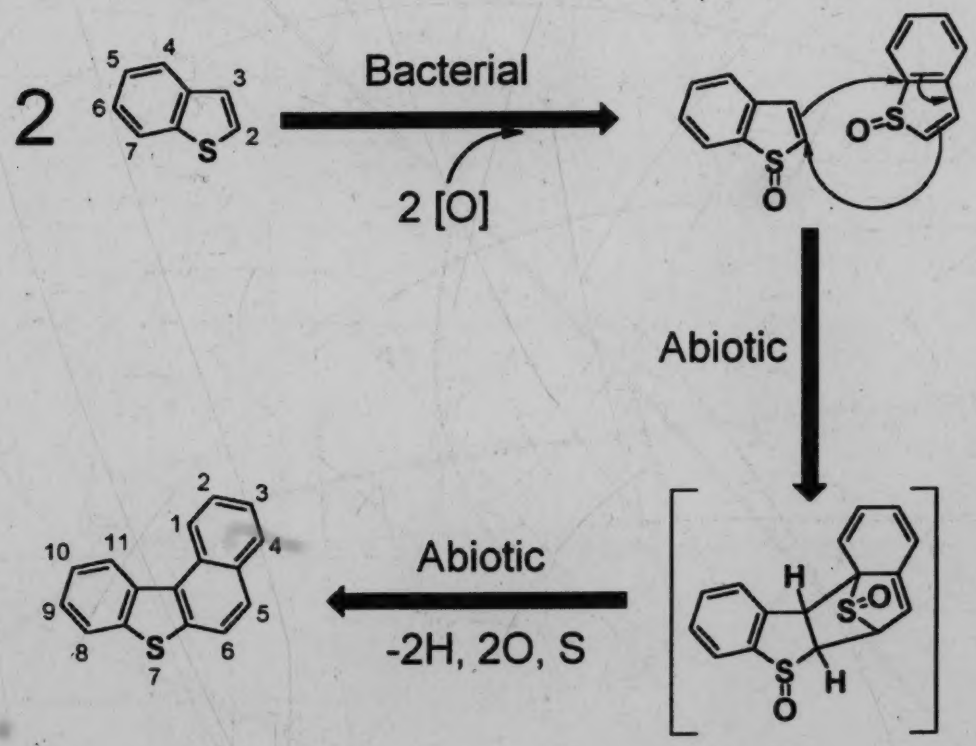


Figure 3.1 Benzonaphthothiophene formed through the Diels-Alder condensation of benzothiophene sulfoxide (as reported by Kropp *et al.* 1994b)

Previous research has shown the initial steps in the biodegradation of dibenzothiophene and benzothiophene produce the oxidized metabolites presented in Figure 3.2 (Kodama *et al.* 1970, 1973; Kropp *et al.* 1994a, 1997; Saftić *et al.* 1992). These metabolites are more polar than the parent compounds and thus are more water-soluble. If the parent compounds are present in the environment and are attacked by

microbes resulting in the release of these polar metabolites, then it is imperative that the further metabolites be identified so that their fates can be determined.

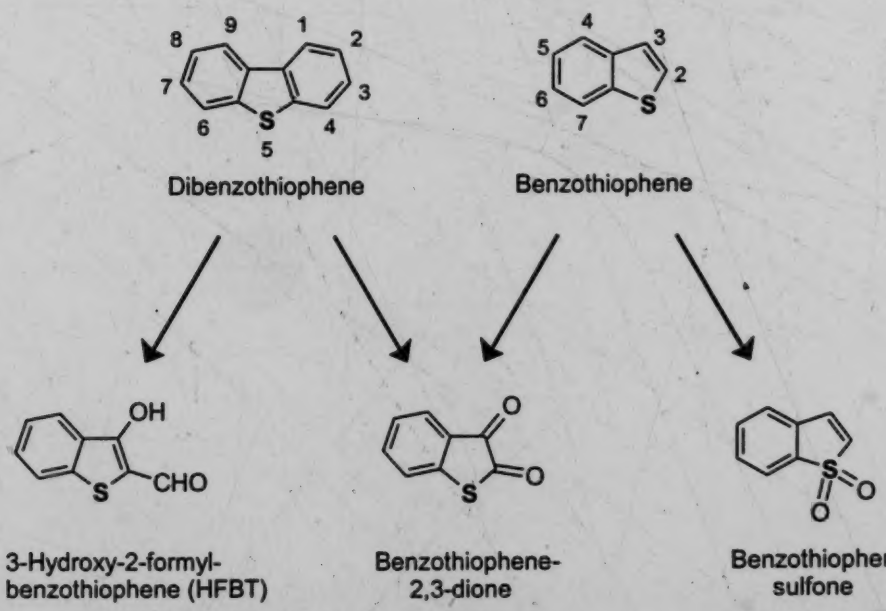


Figure 3.2 Polar oxidized metabolites of benzothiophene and dibenzothiophene (Kodama *et al.* 1970, 1973; Kropp *et al.* 1994a, 1997; Saftić *et al.* 1992).

The results of previous biodegradation studies with benzothiophene sulfones were included as Chapter 2 in this thesis and have been published (Bressler *et al.* 1999). A filamentous bacterium was isolated which utilized benzothiophene sulfone, 3-methylbenzothiophene sulfone, and 5-methylbenzothiophene sulfone as its sole carbon, energy, and sulfur sources. The gene encoding the 16S rRNA was amplified through PCR, sequenced, and then compared to other known sequences in order to classify this filamentous bacterium to the genus *Pseudonocardia*.

Chapter 2 demonstrated that *Pseudonocardia* strain DB1 released up to 44% of the benzothiophene sulfone sulfur as sulfite and sulfate. Similarly, it released 80% and 88% of the sulfur as sulfate and sulfite from the 3-methyl-, and 5-methylbenzothiophene sulfones, respectively. Carbon balances for the biodegradation of the benzothiophene

sulfones were also reported which indicated over half of the biodegraded substrate carbon was released as CO_2 for all three sulfones demonstrating effective mineralization.

Because *Pseudonocardia* strain DB1 was able to effectively mineralize benzothiophene sulfone, 3-methylbenzothiophene sulfone, and 5-methylbenzothiophene sulfone, no biodegradation metabolites were identified in Chapter 2. The investigation reported in this chapter focussed on trying to shorten the observed 7- to 10-day lag period for strain DB1 growth on benzothiophene sulfone by using a heavy inoculum combined with the addition of benzoate to stimulate activity. The investigations also focused on attempts to accumulate metabolites of the sulfones by strain DB1 using a fluorinated analog (5-fluorobenzothiophene sulfone) and a heavy inoculum of strain DB1.

3.2 MATERIAL AND METHODS

3.2.1 Chemicals

Benzothiophene (99%) was purchased from Aldrich (Milwaukee, WI). 3-Methylbenzothiophene (98%) was bought from Lancaster Synthesis (Windham, NH). These compounds were used to synthesize the corresponding sulfones by the method of Bordwell *et al.* (1949). 1-Chloro-2,2-diethoxyethane, 4-fluorothiophenol, polyphosphoric acid and anhydrous chlorobenzene were all purchased from Aldrich. Acetonitrile and dichloromethane (HPLC grade) were from Fisher Chemicals (Fair Lawn, N.J.). Ether was obtained from BDH Inc. (Toronto, ON). N,O-bis-(trimethylsilyl)acetamide was obtained from Pierce (Rockford, IL)

3.2.2 Synthesis of 5-fluorobenzothiophene sulfone

5-Fluorobenzothiophene was synthesized as described by Dupas *et al.* (1993). Briefly, 4-fluorothiophenol was added to a solution of sodium ethoxide (produced by combining elemental sodium and ethanol) and refluxed for 15 h. Following reflux, the ethanol was evaporated, leaving crude 1-(2-diethoxyethylthio)-4-fluorobenzene. The product was redissolved in ethyl ether, washed with water, dried with sodium sulfate, and then concentrated to a residue under reduced pressure.

This crude product was then added slowly to a refluxing solution of polyphosphoric acid and anhydrous chlorobenzene under an argon atmosphere, and refluxed for 24 h after

addition ceased. After cooling, the organic phase containing the product was then separated and pooled with a subsequent dichloromethane extract of the remaining aqueous phase. The 5-fluorobenzothiophene was then purified by silica gel column chromatography with a mobile phase of 80% hexane and 20% dichloromethane. The product was then recrystallized in hexane. Following purification of the 5-fluorobenzothiophene, it was oxidized to the sulfone by the method of Bordwell *et al.* (1949). Purity (>99%) was confirmed by GC-MS analysis.

3.2.3 Effect of 5-fluorobenzothiophene sulfone on the utilization of benzothiophene sulfone by *Pseudonocardia* strain DB1

In an effort to generate detectable levels of metabolites from benzothiophene sulfone by strain DB1, a variety of concentrations of 5-fluorobenzothiophene sulfone were added to DB1 cultures in addition to 100 mg/L of benzothiophene sulfone. Two hundred milliliter cultures of strain DB1 were grown in sulfate-free medium (Bressler *et al.* 1999) in 500-mL shake flask cultures as described in Chapter 2 and by Bressler *et al.* (1999). Cultures were inoculated with 10 mL of a strain DB1 maintenance culture (maintained on 100 mg/L benzothiophene sulfone in sulfate-free medium). The cultures all contained 100 mg/L benzothiophene sulfone and a range of 5-fluorobenzothiophene sulfone concentrations between 0 and 100 mg/L. Utilization of the sulfones was monitored by HPLC.

3.2.4 Sulfone biodegradation with high cell density of strain DB1

Strain DB1 inoculum was prepared by growing strain DB1 in 500-mL Erlenmeyer flasks with 200 mL of sulfate-free medium containing 300 mg/L sodium benzoate. After all benzoate was removed (7-10 days) the cells were harvested by centrifugation at 16300 g for 30 min. The pellet from each flask was washed twice with fresh sulfate-free medium and then recentrifuged to produce washed pellets. Each pellet was then used to inoculate one culture containing 200 mg/L of benzothiophene sulfone, 3-methylbenzothiophene sulfone, or 5-fluorobenzothiophene sulfone with or without 50 mg/L benzoate to induce cell activity.

After 20 days of incubation, the cultures were acidified with 5 mL of concentrated HCl and then extracted three times with 50 mL of dichloromethane. The extracts were dried under reduced pressure in a rotovap and then further concentrated to 100 µL under a nitrogen stream. The resulting extracts were each divided into three portions. The first was analyzed directly using GC with a mass selective detector (GC-MS). The second and third portions were taken to dryness under nitrogen then subsequently derivatized with either diazomethane or N,O-bis-(trimethylsilyl)acetamide (BSA). After derivatization, the samples were again evaporated to dryness under nitrogen and then redissolved in dichloromethane and analyzed by GC-MS.

To further confirm the identity of the metabolite produced during strain DB1 utilization of benzothiophene sulfone, another culture extract was acidified in a similar manner and then lyophilized overnight. The resulting residue was extracted with dichloromethane and then analysed by high resolution GC-MS and GC with a Fourier-transformed infrared detector (GC-FTIR).

3.2.5 Analytical methods

Benzothiophene sulfones were analyzed by HPLC using a Hewlett Packard model 1050 chromatography system and a 125 mm x 4 mm (5 µm) LiChrospher 100 RP-18 column (Hewlett Packard) with a mobile phase of acetonitrile:water (40:60) and flow rates of 1 mL/min, 1.5 mL/min or 2 mL/min. The effluent was monitored at 240 nm with a UV detector.

Fluoride concentrations in culture supernatants were determined in duplicate using an ORION Ion Selective Electrode model 940900.

Low resolution GC-MS was performed using a Hewlett Packard 5890 series II GC with a 5970 series mass selective detector and a 30-m DB-5 capillary column (J&W Scientific, Folsom, CA). High resolution GC-MS was done as outlined previously (Fedorak and Westlake 1986). The GC temperature program used for all of these analyses was 90°C of 1 min, 5°/min to 280°C for 21 min.

The infrared spectrum of the benzothiophene sulfone biodegradation product was obtained by GC-FTIR as previously described by Saftić *et al.* (1993).

3.3 RESULTS

3.3.1 Synthesis of 5-fluorobenzothiophene sulfone

The intermediate product of the synthesis, 1-(2-diethoxyethylthio-)-4-fluorobenzene, was produced with a yield of 26%. The subsequent reactions resulted in the production of 5-fluorobenzothiophene with a yield of 55%. Further purification steps had yields of 60%. The total yield for the entire synthesis was found to be 8%.

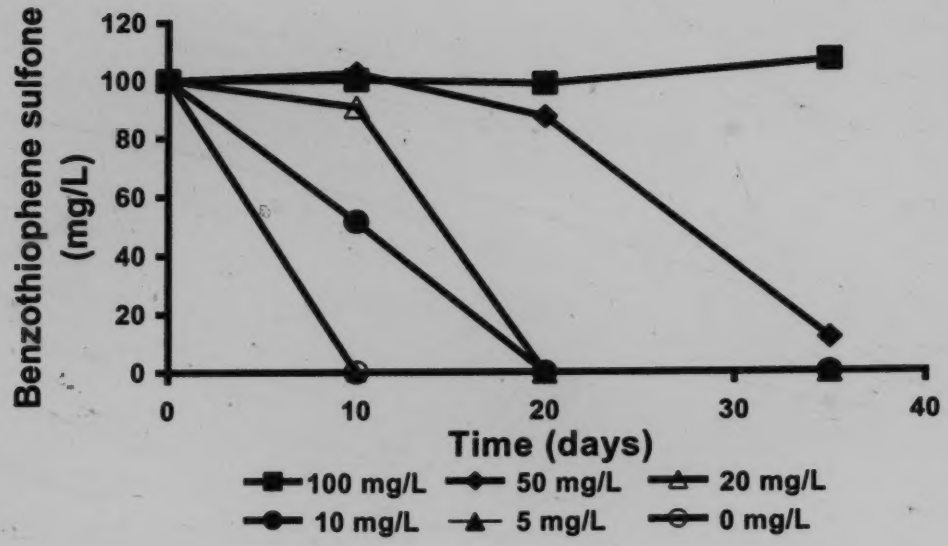
After purification, the synthesis of 5-fluorobenzothiophene sulfone yielded 1.2 g of product that was greater than 99% pure by HPLC and GC analysis.

3.3.2 Effect of 5-fluorobenzothiophene sulfone on the utilization of benzothiophene sulfone by *Pseudonocardia* strain DB1

5-Fluorobenzothiophene sulfone was added to strain DB1 cultures in an effort to generate larger amounts of transient metabolites formed during the biodegradation of benzothiophene sulfone. As shown in Figure 3.3a, as the initial concentrations of the fluorinated sulfone were increased, the lag period before strain DB1 degraded benzothiophene sulfone was increased.

Figure 3.3b shows that 5-fluorobenzothiophene sulfone was also removed from the medium, but not to the extent of the non-fluorinated sulfone (Figure 3.3a). When a 100 mg/L concentration of 5-fluorobenzothiophene sulfone was used, no removal was observed. The apparent increase in concentration during the 35 day incubation could at least partially, if not be completely attributed to concentration due to medium evaporation. Incubations of strain DB1 with 5-fluorobenzothiophene sulfone as the sole substrate demonstrated no growth or utilization of substrate.

a)



b)

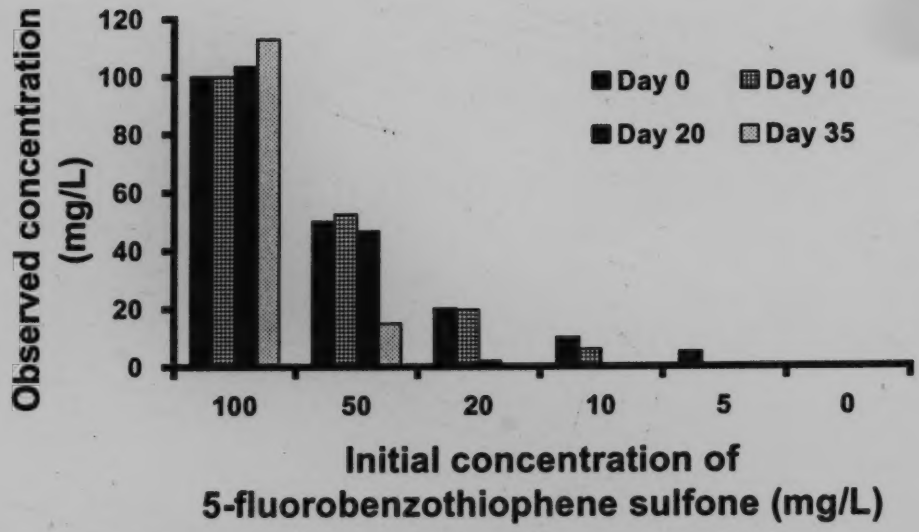


Figure 3.3 a) Strain DB1 utilization of benzothiophene sulfone in the presence of various concentrations of 5-fluorobenzothiophene sulfone.
b) 5-Fluorobenzothiophene sulfone remaining in strain DB1 cultures grown on 100 mg/L benzothiophene sulfone.

Subsequent experiments to generate sulfone biodegradation metabolites were conducted using either 20 or 50 mg/L 5-fluorobenzothiophene sulfone and 100 mg/L benzothiophene sulfone. Figure 3.4 again illustrates that a higher concentration of the fluorinated sulfone results in a prolonged lag period before benzothiophene sulfone utilization. These observations were confirmed through replicates and similar experiments which demonstrated similar trends, but actual numbers varied slightly due to difficulties in producing consistent inoculum. The observed 50 mg/L concentration of 5-fluorobenzothiophene sulfone resulted in an approximately 17 day lag period whereas cultures with 20 mg/L concentration are observed to only have a 10 day lag period. It was also evident that the fluorinated sulfone was utilized simultaneously with the non-fluorinated sulfone. After 1 month of incubation, the culture fluid was analyzed for fluoride. Table 3.1 shows that fluoride was released from 5-fluorobenzothiophene sulfone. Approximately 37% of the fluorine in the substrate was detected as fluoride.

Table 3.1. Release fluoride from 5-fluorobenzothiophene sulfone when incubated with benzothiophene sulfone (100 mg/L) in sulfate-free mineral salts medium in the presence of *Pseudonocardia* strain DB1. (All results are from duplicate cultures after 1 month incubation).

	5-Flurobenzo-thiophene sulfone concentration (mg/L)	Fluoride present (mg/L)	Fluoride if 100% released (mg/L)	Fluorine released as fluoride
Control	50	<0.05	5.1	<1%
Strain DB1 culture	50	1.9	5.1	37%
Control	20	<0.05	2	<2.5%
Strain DB1 culture	20	0.8	2	38%

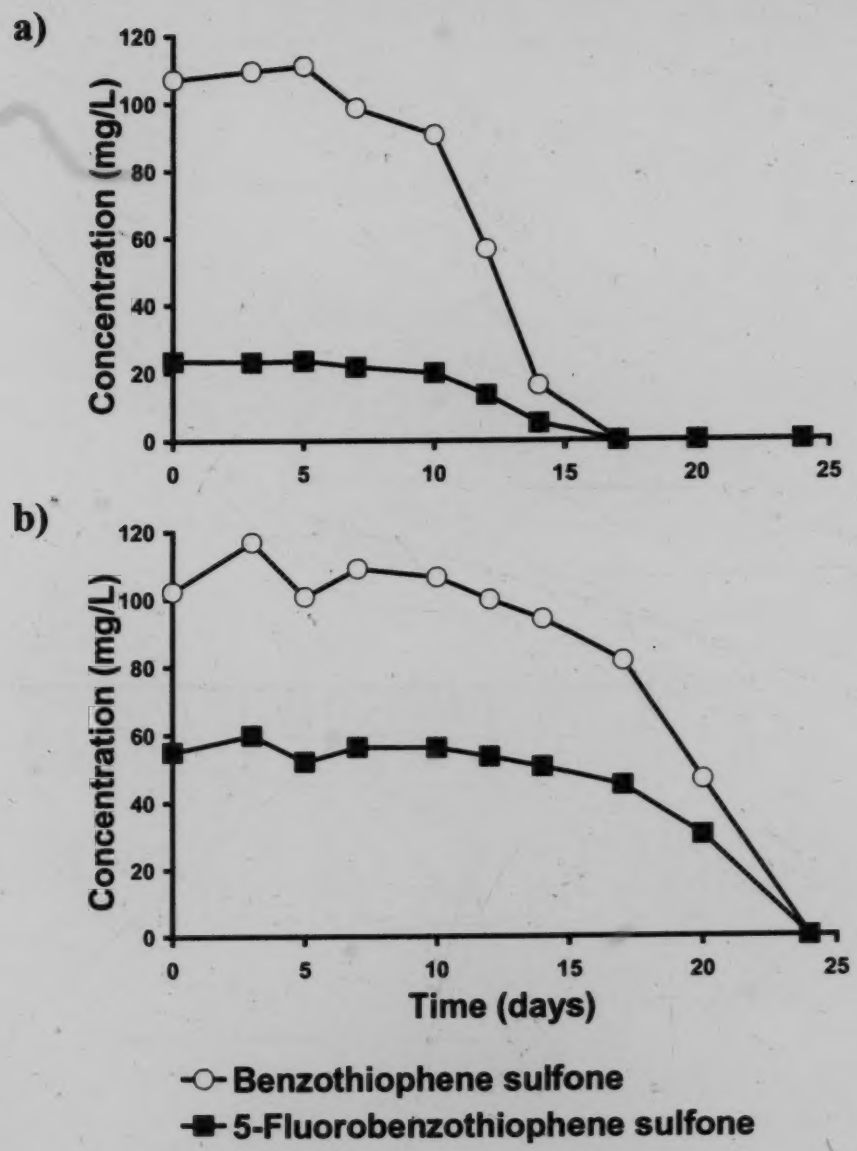


Figure 3.4 The degradation of benzothiophene sulfone and co-metabolism of 5-fluorobenzothiophene sulfone by strain DB1 at different initial concentrations of the latter sulfone (20 mg/L (a) and 50 mg/L (b)) in two different cultures. Corresponding controls demonstrated no substrate loss over the course of the experiment (not shown).

3.3.3 Sulfone utilization in cultures with high cell density of strain DB1

The use of heavy inoculations of strain DB1 were observed to substantially decrease the lag period that strain DB1 had for growth on the sulfones. As shown in Figure 3.5, the lag period for utilization of the sulfones was less than 4 days. Benzoate was included as an experimental variable because it was observed in preliminary experiments that the use of benzoate as a carbon and energy source could induce strain DB1 growth with very little lag period.

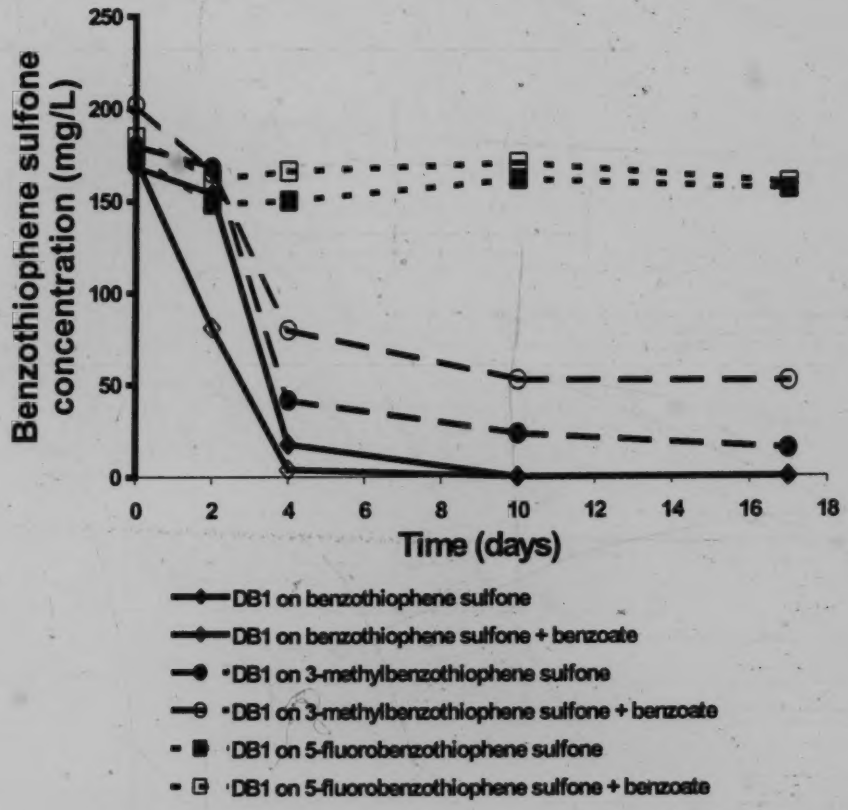


Figure 3.5 Degradation of benzothiophene sulfones in cultures with high cell density of *Pseudonocardia* strain DB1. All cultures initially contained 200 mg/L of the respective sulfone. In all active cultures, benzoate was observed to be degraded by day 2. Sterile controls did not show significant substrate loss over time (not shown).

It was thought that adding benzoate could initiate cell activity and once benzoate was consumed, initiation of sulfone biodegradation would occur faster than without induction. Previous studies on strain DB1 sulfone utilization (Chapter 2) revealed lag periods of no less than 7 days when less concentrated inoculum was used. With heavy inoculum (Figure 3.5), strain DB1 was able to degrade a small proportion of the 5-fluorobenzothiophene sulfone, with or without the use of benzoate as an inducer, resulting in the medium turning a faint brown color. This ability was not observed with less concentrated inocula. Overall the use of additional benzoate as a primary substrate only slightly decreased the lag periods seen for benzothiophene sulfone biodegradation, and did not appear to be utilized simultaneously with any of the sulfones as observed in Figure 3.5. During this experiment, it was observed that benzoate was removed from all active cultures by day 2 yet sulfone degradation occurred after that time. This observation indicates that strain DB1 first utilized the benzoate and then switched its metabolism to utilize the sulfones instead of utilizing both substrates simultaneously.

3.3.4 Identification of sulfone biodegradation metabolites

Culture extracts of heavily inoculated strain DB1 grown on the various sulfones for 20 days were acidified and extracted with dichloromethane. Subsequent analyses of these extracts revealed identifiable products from benzothiophene sulfone and 5-fluorobenzothiophene sulfone.

Benzothiophene sulfone biodegradation by strain DB1 produced one major metabolite with a molecular weight of 184 observed by GC-MS (Figure 3.6). High resolution GC-MS revealed that this metabolite had a molecular formula of $C_8H_8SO_3$. Subsequent GC-FTIR analysis (Figure 3.7) revealed the presence of a hydroxyl group (3581 cm^{-1}) and characteristic sulfone peaks as well (1348 cm^{-1} and two peaks at 1195 cm^{-1} due to steric difference between the two sulfone oxygen atoms). Analysis of the mass spectrum (Figure 3.6) demonstrates a fragmentation pattern revealing a loss of OH (184 - 17 = mass 167) and high resolution GC-MS shows the remaining molecular formula to be $C_8H_7SO_2$. To confirm the presence of the hydroxyl group, portions of the extract were derivatized with either diazomethane or BSA. Diazomethane derivatization did not affect the metabolite as observed by GC-MS analysis. BSA derivatization

resulted in the removal of the metabolite peak and produced a peak with a mass of 256. Derivatization with BSA but not diazomethane strongly indicated the production of the trimethylsilyl derivative, characteristic of the presence of a hydroxyl group.

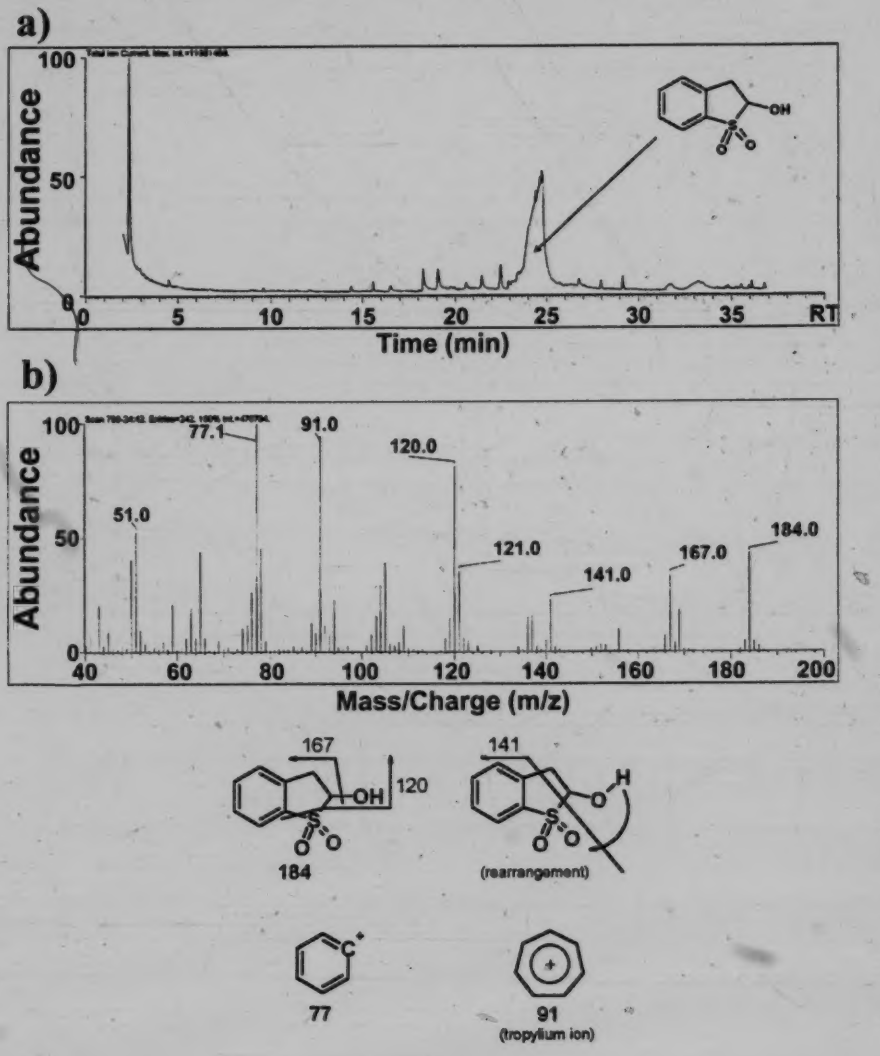


Figure 3.6 a) GC-MS total ion current and b) mass spectrum of the metabolite (24 min peak) produced from benzothiophene sulfone by strain DB1.

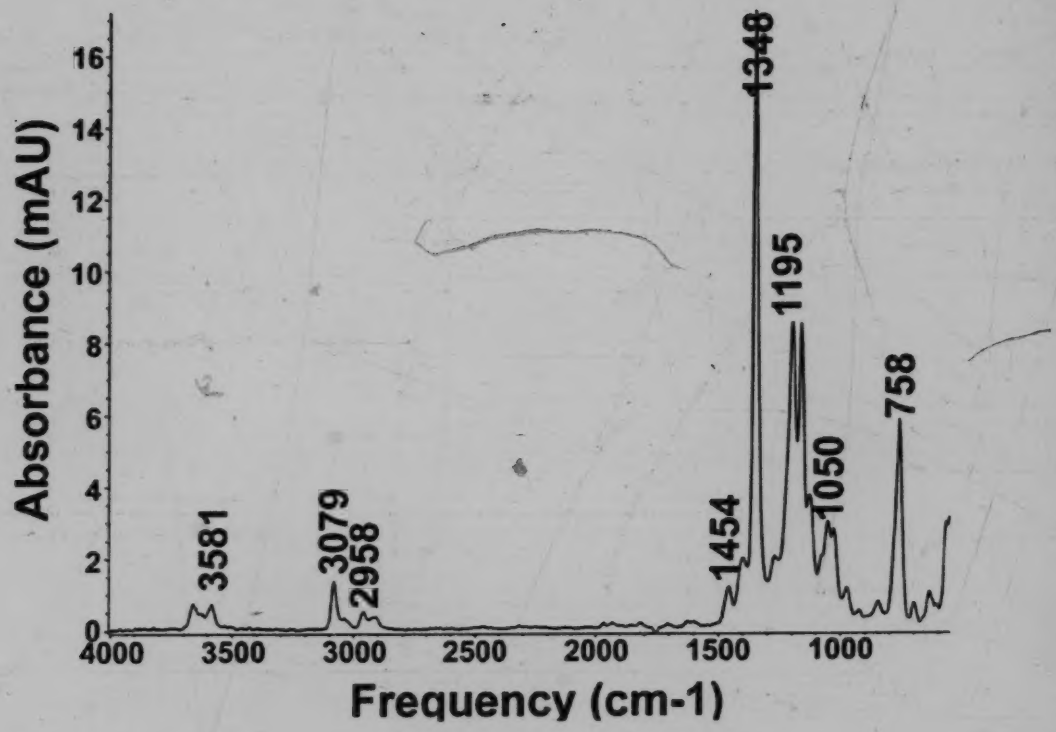


Figure 3.7 Infrared spectrum of benzothiophene sulfone strain DB1 biodegradation product obtained through GC-FTIR analysis.

The mass spectrum (Figure 3.6b) also demonstrates the presence of a strong tropylium ion (mass 91 and formula C_7H_7), produced by having a CH_2 group adjacent to a benzene ring. Figure 3.8 demonstrates literature spectra for phenylethyl alcohol and 1-phenylethanol, two compounds closely related to each other differing only in the location of the alcohol group. It can be observed that the tropylium ion (mass 91) is only formed when there is a CH_2 group adjacent to the benzene ring (Figure 3.8a). The molecular formula of the unknown compound in combination with the knowledge that both a sulfone group and a hydroxy group are present and the ability to form the tropylium ion exists, suggested that the metabolite was dihydrohydroxybenzothiophene sulfone (Figure 3.6a). To form the tropylium ion, the CH_2 group must be in the 3 position which indicates the thiophene ring must be saturated at both the 2, and 3 positions. The only

two possible positions for the hydroxyl group would be at the 2 position or on the benzene ring. If the mass spectrum is analyzed closer (Figure 3.6b) it is apparent that a fragment with a mass of 141 is formed and high resolution GC-MS confirms the molecular formula of this peak to be $C_6H_5SO_2$. As diagrammed in Figure 3.6 the most likely structure to give this fragment is if the hydroxyl group is found in the 2 position. It is also highly unlikely that in a bacterial biodegradation system that the enzymes and any abiotic reactions involved would saturate the thiophene ring and then oxidize the benzene ring leaving a phenol. For these reasons, the proposed structure for the identified metabolite is 2,3-dihydro-2-hydroxybenzothiophene sulfone (Figure 3.6).

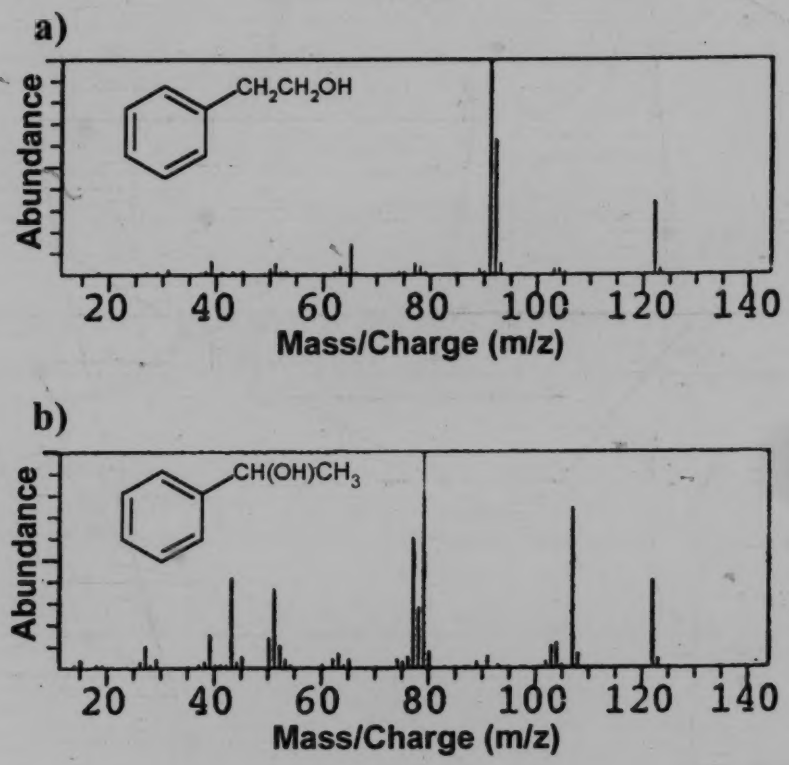


Figure 3.8 Reference mass spectra of (a) phenylethyl alcohol and (b) 1-phenylethanol. Note the presence of a strong tropylium ion (mass 91) in the phenylethyl alcohol spectrum due to the CH_2 group adjacent to the benzene ring, but not in the 1-phenylethanol spectrum (CHOH adjacent to benzene ring). (Taken from McLafferty and Stauffer 1989)

Analysis of culture supernatants of heavily inoculated strain DB1 cultures biodegrading small amounts of the 5-fluorobenzothiophene sulfone demonstrated the production of a product with a mass of 202 (Figure 3.9b).

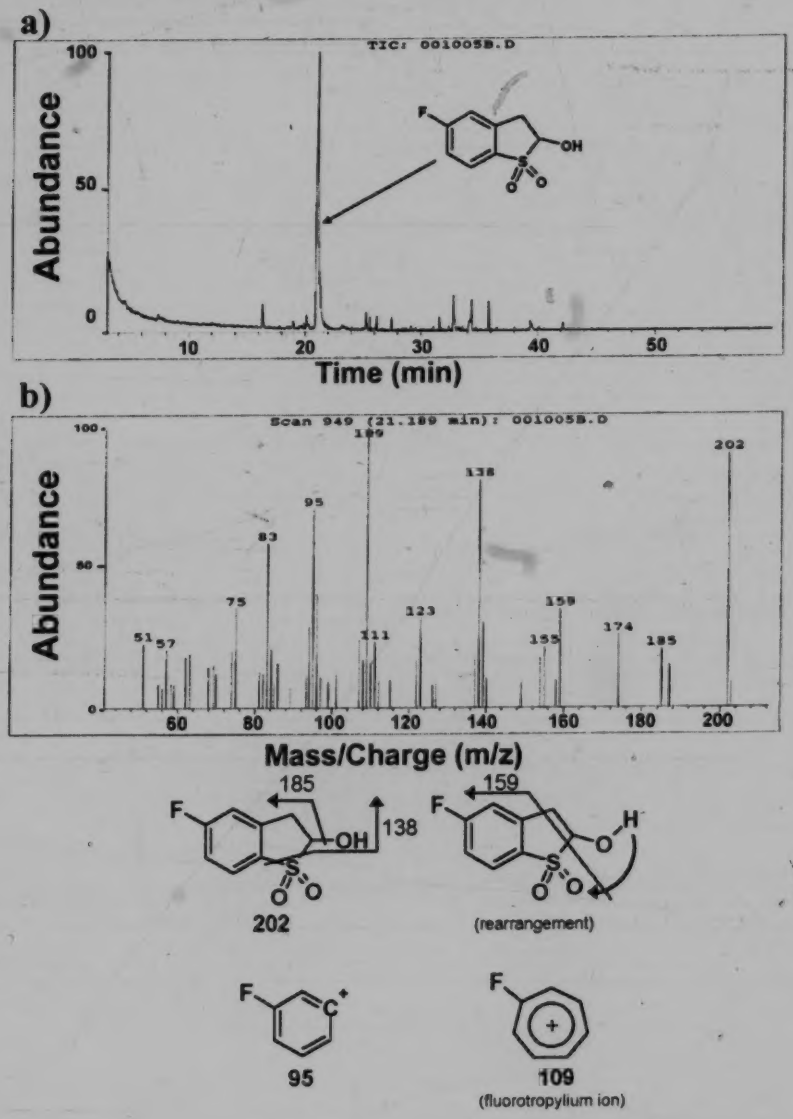


Figure 3.9 GC-MS analysis and mass spectrum of the metabolite (21.2 min peak) produced from 5-fluorobenzothiophene sulfone by strain DB1.

Upon analysis of the mass spectrum, the metabolite appeared to be 5-fluoro-2,3-dihydro-2-hydroxybenzothiophene sulfone as shown in Figure 3.9a. The mass spectrum was very similar to that of the metabolite from benzothiophene sulfone, except that it demonstrated the addition of a fluorine atom to the benzene ring (demonstrated by the tropylium ion having a mass of 109 and the benzene ring fragment having a mass of 95). Again diazomethane derivatization did not affect the metabolite as observed by GC-MS and BSA resulted in derivatization of the metabolite.

Experiments with 3-methylbenzothiophene sulfone demonstrated complete substrate removal, but an analogous metabolite could not be detected. A selective mass scan on the GC-MS revealed a complete absence of the expected 198 mass. Subsequent diazomethane and BSA derivatizations revealed no identifiable peaks.

3.3.5 Diels-Alder condensation products

HPLC analysis of incubations of benzothiophene sulfone with *Pseudonocardia* strain DB1 revealed the presence of a compound with a retention time three to four times longer than the sulfone. During the course of extracting and performing routine GC-MS analysis described in Chapter 2, no metabolite with a large molecular weight was observed. A new GC temperature program was employed which involved a more rapid heating of the column to a higher temperature (280°C versus 250°C previously). This new temperature program revealed the presence of two products which were shown to be present in all cultures after incubation and in the purified benzothiophene sulfone crystals as well. The ratio of the condensation products to the benzothiophene sulfone appeared qualitatively to be higher in both sterile and active cultures after incubation than in the original crystals, but quantitation was not attempted. The two compounds were found to have molecular weights of 266 and 268 (Figure 3.10). GC-FTIR analysis was unable to separate the two compounds, but it revealed that the only recognizable functional group present in both compounds was a sulfone. It could also be observed (Figure 3.11) that a small proportion of non-aromatic protons were present as well as demonstrated by C-H absorptions below 3000 cm^{-1} (2920 cm^{-1}). Based on these molecular weights and functional group assessments, the compounds are suggested to be benzonaphthothiophene sulfone and the dihydroxybenzonapthothiophene sulfone shown in Figure 3.10. It is

believed that these condensation products were formed through the abiotic condensation of two benzothiophene sulfone molecules during benzothiophene sulfone preparation and during culture incubations. These condensation products were not observed to be present in any of the substituted benzothiophene sulfone preparations or cultures.

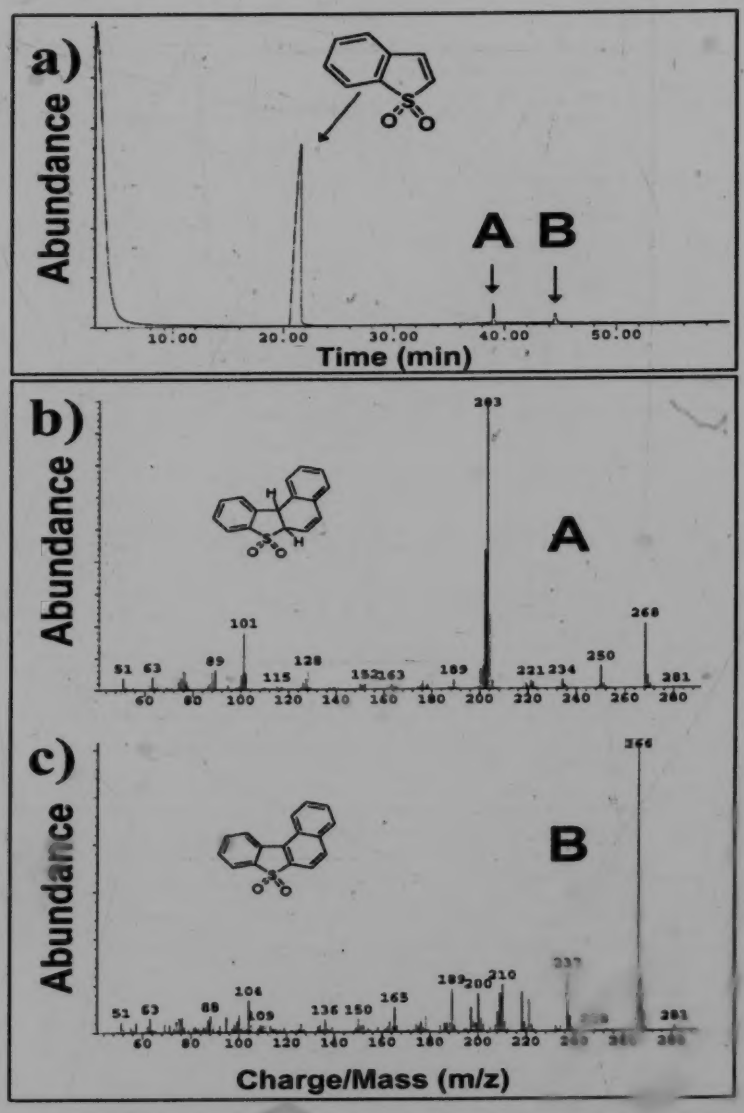


Figure 3.10 GC-MS analysis of benzothiophene sulfone crystals dissolved in dichloromethane.

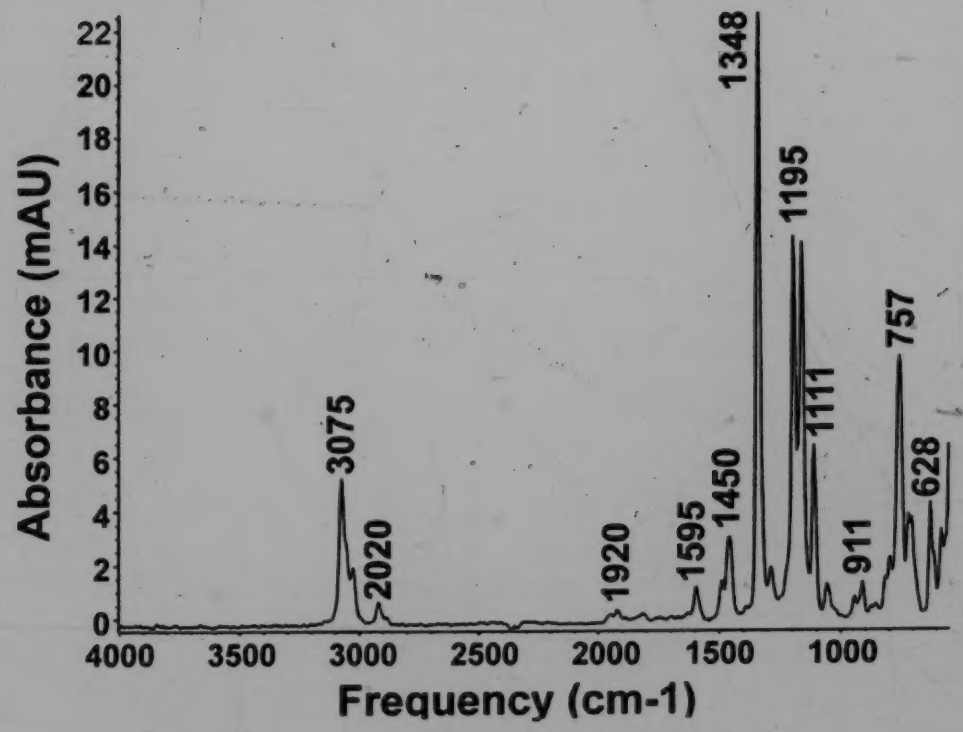


Figure 3.11 GC-FTIR spectrum of condensation product formed through a Diels-Alder condensation of two molecules of benzothiophene sulfone.

3.4 DISCUSSION

This investigation focused on detecting metabolites from the biodegradation of benzothiophene sulfones by *Pseudonocardia* strain DB1. Chapter 2 (Bressler *et al.* 1999) clearly demonstrated that strain DB1 was very proficient at biodegrading benzothiophene sulfone, 3-methylbenzothiophene sulfone, and 5-methylbenzothiophene sulfone. Unfortunately, during the earlier study, metabolites of sulfone degradation were not observed, presumably due to the efficiency of strain DB1 mineralization. This Chapter focused on both the generation of larger quantities of metabolites, allowing identification, and the reduction of strain DB1 lag periods. Two main approaches were employed to this end. The first approach focused on the use of benzoate as an inducer and heavily

inoculated washed cell suspensions. The second approach focused on the use of the fluorinated analog 5-fluorobenzothiophene sulfone. It was anticipated that the fluorinated analog would either be partially biodegraded, resulting in an accumulating metabolite, or would potentially interfere with benzothiophene sulfone biodegradation if added to biodegrading cultures.

It was observed that benzoate served as a good carbon and energy source for strain DB1. When grown on benzoate alone, strain DB1 displayed a lag period of only 24-48 h which was much shorter than the lag for growth on the sulfones alone (7-10 days). Strain DB1 could also be incubated with both a sulfone and benzoate resulting in diauxic growth, first utilizing the benzoate with little lag, and then metabolizing the available sulfone. This diauxic growth was able to decrease the lag period for benzothiophene sulfone utilization as compared to sulfone alone. Thus the goal of decreasing strain DB1 lag periods for sulfone utilization was partially accomplished.

To further decrease the lag period for sulfone utilization and to try to generate significant quantities of sulfone metabolites, washed cell suspensions, grown on benzoate, were used to heavily inoculate sulfone-containing cultures to a high cell density. The combination of benzoate as an inducer and high cell densities of strain DB1 decreased the lag period for sulfone utilization to 24-48 h which was a significant reduction from the 7 to 10 days observed in previous experiments (Chapter 2). Benzoate may not act as an inducer of genes necessary for sulfone biodegradation, but instead simply act to initiate the exponential growth phase. The large lag periods apparent for growth of strain DB1 on benzothiophene sulfone may indicate that the sulfones do not directly induce genes necessary for their biodegradation. It is suggested that perhaps very low constitutive levels of biodegradative enzymes are present in strain DB1. These enzymes would be responsible for the initial attack on benzothiophene sulfone resulting in an oxidized metabolite, which may then activate the genes necessary for sulfone utilization. Due to the low levels of this initial biodegradative enzyme, it would take time for the concentration of an oxidized metabolite to accumulate to a concentration high enough to induce higher level expression of the biodegradative genes necessary for sulfone biodegradation.

The second goal of this investigation was to identify metabolites of sulfone biodegradation. 5-Fluorobenzothiophene sulfone was incorporated into experiments as either a potential sole carbon, energy, and sulfur source, or as a co-substrate with benzothiophene sulfone. The biodegradation of fluorinated aromatic compounds has been previously studied to some extent (Neilson 2000), but a recent literature search revealed no studies with aromatic compounds larger than benzoate or with heterocyclic compounds. Indications are that the use of fluoroaromatic compounds as sole carbon and energy sources may be restricted such that they can only be degraded under conditions of concurrent metabolism where a suitable growth substrate is provided (Neilson 2000). Studies involving fluorinated analogs have generally demonstrated defluorination and have implicated dioxygenase and hydroxylase enzymes as being capable of this activity (Renganathan 1989; Husain *et al.* 1980). Studies have not focused on the generation of aerobic metabolites from non-fluorinated compounds by using fluorinated analogs to interfere with substrate metabolism. This investigation employed a fluorinated analog to try to generate metabolites from a non-fluorinated heterocycle during utilization of both substrates simultaneously.

Anaerobic biodegradation of fluoroaromatic compounds has been studied less intensely, and the investigations reported do not report defluorination (Neilson 2000). However, Londry and Fedorak (1993) successfully utilized fluorinated analogs to generate identifiable metabolites during the study of *m*-creosol degradation by a methanogenic consortium.

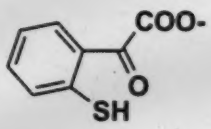
This investigation clearly demonstrated that 5-fluorobenzothiophene sulfone could only be biotransformed when benzothiophene sulfone was supplied as the primary carbon and energy source. There was evidence however, that defluorination of the fluorinated analog was occurring to some extent as witnessed by the release of significant amounts of fluorine as fluoride. It was also observed that 5-fluorobenzothiophene sulfone interfered with the biodegradation of benzothiophene sulfone. Increasing the initial concentration of the fluorinated sulfone resulted in greater inhibition and longer lag periods for the utilization of benzothiophene sulfone by strain DB1. These findings support the belief that these fluorinated analogs do not effectively serve as sole carbon and energy sources, but that defluorination is possible under aerobic conditions. One area of investigation

that may prove successful would be to follow the biotransformation of 5-fluorobenzothiophene sulfone using ^{19}F-nuclear magnetic resonance. This technique would allow the tracking of the oxidation reactions involved in the biodegradation of the fluorinated sulfone. These types of ^{19}F-nuclear magnetic resonance experiments tracking the biodegradation of fluorinated compounds have been done before with the most recent example being that of Prenafeta-Boldú *et al.* (2001). In their investigation, Prenafeta-Boldú *et al.* (2001) investigated the use of isomeric fluorotoluenes as model substrates to study the catabolism of toluene by five deuteromycete fungi and one ascomycete fungus capable of growth on toluene as the sole carbon and energy source. By using these types of experiments it may be possible to determine if attack on the sulfone by strain DB1 occurs at the thiophene ring, the benzene ring, or randomly at either ring.

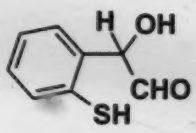
Studies utilizing the 5-fluorobenzothiophene sulfone and benzothiophene sulfone with concentrated washed cell inoculum resulted in the identification of 5-fluoro-2,3-dihydro-2-hydroxybenzothiophene sulfone and 2,3-dihydro-2-hydroxybenzothiophene sulfone as metabolites. From the data it could not be determined if they were transient or dead end metabolites. Of interest was that 3-methylbenzothiophene was clearly biodegraded by strain DB1 without forming the corresponding dihydrohydroxymethylbenzothiophene. A literature search has revealed that there have been no studies involving the biodegradation, toxicity, synthesis, or other chemical properties of the 2,3-dihydro-2-hydroxybenzothiophenes or the benzonaphthothiophene sulfones. Further investigations are required to accurately determine the environmental implications of these metabolites if they formed through biodegradative processes in the environment.

It is possible that strain DB1 is able to utilize two different metabolic attack pathways for the biodegradation of benzothiophene sulfones. The first pathway which is supported by the identification of the 2,3-dihydro-2-hydroxybenzothiophenes would be the result of oxidative attack on the thiophene ring component of benzothiophene sulfones. A second attack pathway could be initiated by dioxygenase attack on the benzene ring of the sulfones. Supporting evidence for this hypothesis can be found in Chapter 2 and in the literature (Eaton and Nitterauer 1994). In Chapter 2, it was observed that sulfate release was not equivalent for biodegradation of the 3-

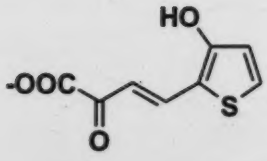
methylbenzothiophene sulfone, 5-methylbenzothiophene sulfone, and benzothiophene sulfone. The methylbenzothiophene sulfones were observed to release 77% and 88% of their sulfur as sulfate, while biodegradation of benzothiophene sulfone was observed to release 44% only of the sulfur as sulfate. It is possible that benzothiophene sulfone biodegradation was evenly split between the two attack pathways while the presence of the methyl substituents resulted in the selection of the sulfate releasing attack pathway over the other. Eaton and Nitterauer (1994) investigated the biodegradation of the parent condensed thiophene benzothiophene. In their investigation, they report the identification of a variety of metabolites resulting from initial cleavage of the thiophene ring or the benzene ring in benzothiophene. The assortment of ring cleavage products they reported included 2-mercaptophenylglyoxylate, 2'-mercaptomandelaldehyde, *trans*–4-[3-hydroxy-2-thienyl)-2-oxobut-3-enoate and 3-hydroxythiophene-2-carboxaldehyde (Figure 3.12). Clearly there exists the possibility of two distinct biodegradation pathways for benzothiophene and benzothiophene sulfones. Further investigations are required to fully understand the role substituents play in the selection of biodegradation pathways for the benzothiophene sulfones.



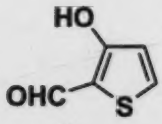
2-Mercaptophenylglyoxylate



2'-Mercaptomandelaldehyde



trans-4-[3-Hydroxy-2-thienyl)
-2-oxobut-3-enoate



3-Hydroxythiophene
-2-carboxaldehyde

Figure 3.12 Biodegradation products of benzothiophene reported by Eaton and Nitterauer (1994)

Previous investigations by Kropp et al. (1994b) revealed the presence of benzonaphthothiophenes in culture extracts. It was suggested by Kropp *et al.* (1994b) that the benzonaphthothiophenes were strictly products of benzothiophene sulfoxide Diels-Alder condensation reactions. This investigation revealed the presence of benzonaphthothiophene sulfones in the benzothiophene sulfone preparation. The apparent increasing concentration of these condensation products may indicate that this condensation also occurs during culture incubation and that it is the result of the condensation of two benzothiophene sulfone molecules. This investigation has revealed two more types of condensation reactions formed from the biodegradation of benzothiophene sulfones. It would be of interest to look for the presence of these metabolites in bacterial cultures known to form benzothiophene sulfone during benzothiophene biodegradation.

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4. PURIFICATION, STABILITY AND MINERALIZATION OF 3-HYDROXY-2-FORMYLBENZOTHIOPHENE, A METABOLITE OF DIBENZOTHIOPHENE *

4.1 INTRODUCTION

For more than 4 decades, dibenzothiophene (DBT) has been the model compound for studying the biodegradation or biodesulfurization of sulfur heterocycles. 3-Hydroxy-2-formylbenzothiophene (HFBT) has often been found as a metabolite of bacterial degradation of DBT (Bohnos *et al.* 1977; Frassinetti *et al.* 1998; Kodama *et al.* 1970, 1973; Laborde and Gibson 1977). Yamada *et al.* (1968) reported that two *Pseudomonas* isolates produced a metabolite that upon purification gave of a yellow crystal with a melting point of 107°C. Kodama *et al.* (1970) conclusively identified this metabolite as HFBT (Figure 4.1a).

Figure 4.1b shows that two molecules of HFBT can undergo a spontaneous abiotic reaction forming a red "dye-stuff", 3-oxo-(3'-hydroxythianaphthenyl-2-methylene)-dihydrothianaphthene (III) (Kodama *et al.* 1970, 1973). Finkel'stein *et al.* (1997) described a different product of two molecules of HFBT spontaneously reacting to give 3,3'-dihydroxy-2,2'-dithianaphthene (IV Figure 4.1b). However, they based this tentative identification solely on the interpretation of a mass spectrum.

No biodegradation products of HFBT have been conclusively identified, but compounds that may originate from HFBT have been detected in cultures biodegrading DBT. Examples of these are shown in Figure 4.2. Bohonos *et al.* (1977) found 3-hydroxybenzothiophene (V), and benzothiophene-2,3-dione (VIII). Eaton and Nitterauer (1994) showed that when acidic conditions are used to extract culture supernatants containing 2-mercaptophenylglyoxalate (VII), an acid-catalyzed dehydration occurs to form benzothiophene-2,3-dione (VIII). Finkel'stein *et al.* (1997) detected 2,3-dihydroxybenzothiophene (VI), and 2-mercaptobenzoic acid (or thiosalicylic acid, IX). However, no one has conclusively shown that these metabolites result from further catabolism of HFBT and are not products of a concurrent metabolic pathway.

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* A version of this chapter has been previously published.
Bressler, D.C., and P.M. Fedorak. 2001. Appl. Environ. Microbiol. 67:821-826.

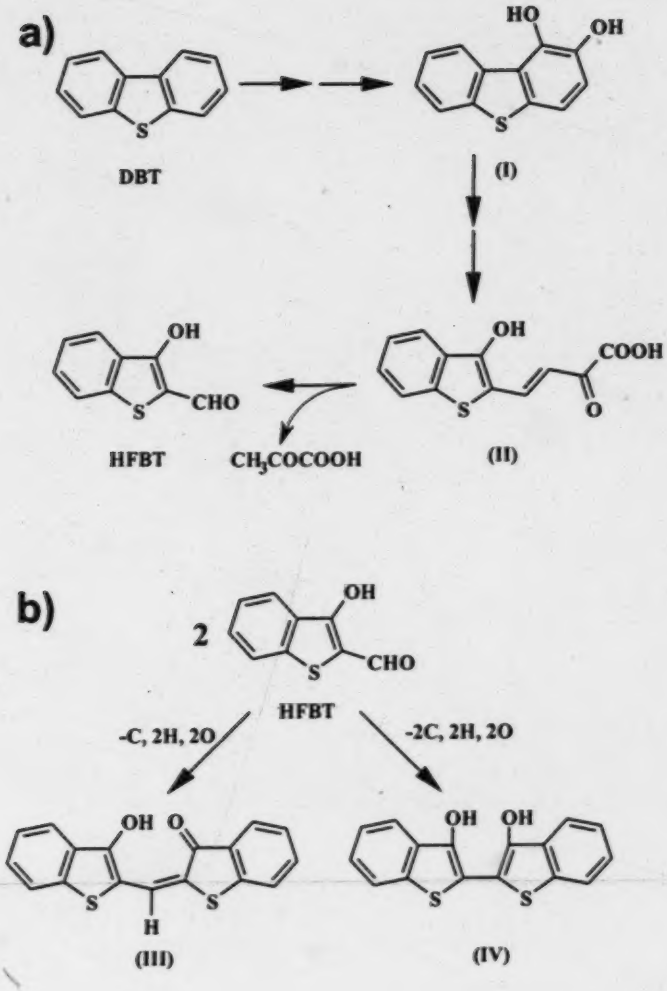


Figure 4.1 Abbreviated Kodama pathway for DBT biodegradation to HFBT (a) and proposed products from HFBT and references in which they were reported (b). (I) 1,2-dihydroxydibenzothiphene (Laborde and Gibson 1977); (II) *trans*-4[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid (Kodama *et al.* 1973); (III) 3-oxo-(3'-hydroxythianaphthenyl-2-methylene)-dihydrothianaphthene (Kodama *et al.* 1970); (IV) 3,3'-dihydroxy-2,2'-dithianaphthene (Finkel'stein *et al.* 1997).

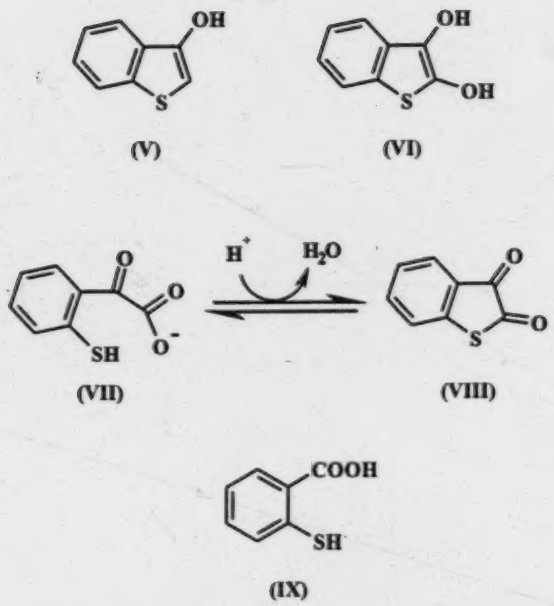


Figure 4.2 Some possible biodegradation products of HFBT and references in which they were reported: (V) 3-hydroxybenzothiophene (Bohonos *et al.* 1977); (VI) 2,3-dihydroxybenzothiophene (Bohonos *et al.* 1977); (VII) 2-mercaptophenylglyoxalate (Eaton and Nitterauer 1994); (VIII) benzothiophene-2,3-dione (Bohonos *et al.* 1977); (IX) 2-mercaptobenzoic acid (Finkel'stein *et al.* 1997).

To date, purified HFBT has not been used as a carbon and energy source for biodegradation studies. The only reported investigation into the biodegradation of HFBT is by Mormile and Atlas (1988) who used filter-sterilized culture supernatants containing HFBT, *trans*-4[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid (II, Figure 4.1a), and presumably other metabolites, produced by a strain of *Pseudomonas putida* growing on dibenzothiophene. These were added to enrichment cultures inoculated with soil or creek sediment samples, and the loss of HFBT was monitored spectrophotometrically at 390 nm, and by measuring CO_2 production. The absorbance at 390 nm decreased to about 40% of its initial value over a 21-day incubation period. CO_2 release was observed, but sulfate release was not detected.

The fate of HFBT in cultures or in the environment is unknown. Laboratory studies to address its fate have been hampered because HFBT is not commercially available, and it has not been produced in sufficient quantities for such investigations. This paper describes experiments used to produce HFBT by chemical and microbiological methods, the chemical stability of HFBT, and the mineralization of HFBT by a mixed microbial culture.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of HFBT by microbiological methods

Pseudomonas strain BT1d grows on DBT as its sole carbon and energy source resulting in the conversion of about 50% of this substrate to HFBT (Kropp and Fedorak 1998). For production of HFBT, this strain was inoculated into several 2-L Erlenmeyer flasks that each contained 1.5 L of mineral medium (Kropp *et al.* 1994a) and 2 g of DBT (most of which remained as crystals). These cultures were incubated at 28°C with shaking for 2 months. Then the culture supernatant was acidified with citric acid (Aldrich Chemical Company, Milwaukee, WI) to pH~2, and extracted three times with 200-mL portions of ethyl acetate. Citric acid was used because it would not extract into the ethyl acetate and mineral acids such as HCl or H_2SO_4 accelerated the loss of HFBT. It was also found that the use of dichloromethane as an extraction solvent increased the loss of HFBT by an unknown mechanism. The ethyl acetate extracts were combined and all the solvent was removed at 50°C under vacuum in a rotary evaporator.

The resulting HFBT-containing residue was immediately purified by sublimation yielding yellow HFBT crystals. The sublimation apparatus consisted of a 1-L three-necked, round-bottom flask which contained the dried crude HFBT preparation. This flask was connected to a vacuum pump through one neck of the flask. A coldfinger with a funnel top was then connected to the center neck of the flask and filled with a dry ice ethanol mixture. The third flask opening was sealed. The flask was then pumped to vacuum and submerged in a paraffin oil bath which was heated to 80°C by a hotplate with stirring. The HFBT was allowed to sublime for approximately 4 h, which was sufficient to achieve close to maximum yield of yellow HFBT crystals.

Steam distillation was also evaluated as a method for HFBT purification from the culture supernatant after acidifying it to pH 2 with HCl. Approximately 200 mL of the steam distillate was collected in a round bottom flask which contained 10 mL of 0.1 mM phosphate buffer (pH 7).

4.2.2 Chemical synthesis of HFBT

The chemical synthesis of HFBT was conducted as described by Smiles and McClelland (1921). The synthesis involved heating a mixture of malic acid, thiosalicylic acid (both obtained from Aldrich) and concentrated sulfuric acid to 90°C for 30 min. Unfortunately HFBT was not the major product. Purification of the HFBT was attempted by sublimation and by steam distillation.

4.2.3 Abiotic reactions of HFBT in crystalline form

Crystals of HFBT slowly turned from yellow to a reddish purple color when stored in a sealed vial with air in the headspace. To examine these spontaneous reaction products, crystals that had been stored for 1 year were dissolved in dichloromethane. A portion of this solution was analyzed by gas chromatograph-mass spectrometry (GC-MS), and another portion was analyzed by preparative thin layer chromatography (TLC) on 20 x 20 cm plates with a 250 μm layer of 60 Å K6F silica gel (Whatman Inc., Clifton NJ). The plate was developed with a mixture of hexane and ethyl acetate (70:30). The two pink bands were then scraped off the plate and washed from the silica gel with dichloromethane. The extract was then analyzed by low resolution and high resolution GC-MS. One of the pink compounds, with the R_f of 0.82 was analyzed by Fourier transformed infrared spectroscopy.

A purple band remained at the origin of the TLC plate. A solvent mixture containing dichloromethane, methanol and hexane (45:10:45) was required to move this compound from the origin. The purple compound was then scraped and eluted into dichloromethane for analysis.

4.2.4 Stability of HFBT in sterile aqueous solutions

Aerobic shake-flask incubations were conducted in the dark at 28°C with 0.07 mM HFBT in a variety of different aqueous solutions (200 mL) in 500-mL Erlenmeyer flasks. One set of three flasks contained Milli-Q water (pH ~7), dilute HCl (pH 2), and dilute NaOH (pH 11). Another set of three flasks contained sulfate-free medium (Bressler *et al.* 1999) adjusted pH 7, 2 or 11. Another aerobic shake flask contained HFBT in sulfate-free medium (pH 7) and was incubated with constant direct light (100 W light bulb from 0.2 m distance). Finally, an anaerobic incubation was done in a sealed flask with a N_2 headspace and 1 mM sodium sulfide added to the sulfate-free medium. These were all incubated for 55 days, with samples being removed at various times for high performance liquid chromatography (HPLC) analyses for residual HFBT.

4.2.5 Biodegradation and mineralization of HFBT

A mixed culture, designated SLPB, that has been maintained in liquid culture on Prudhoe Bay crude oil since 1983 (Fedorak and Peakman 1992) was used to enrich the HFBT-degrading culture. Twenty milliliters of SLPB were transferred to 200 mL of mineral medium with 10 mg of HFBT as the sole carbon and energy source. This shake-flask culture was incubated at 28°C for 3 weeks. Then 20 mL of this enrichment culture were transferred to fresh mineral medium with HFBT and incubated under the same conditions. This was repeated five times over a 4-month period. At various times, these enrichment cultures were analyzed for the decrease in HFBT content by HPLC.

The final enrichment culture was used for the mineralization study in which 10 mL of the culture were added to 70 mL of mineral medium with 7 mg (0.039 mmol) HFBT in sealed 160-mL serum bottles. Triplicate viable cultures and triplicate heat-killed, sterile controls were prepared. These cultures were incubated at 28°C and shaken at 200 rpm. The headspace gas contained sufficient O_2 for complete mineralization of the HFBT. After 14 days incubation, 2 mL of 5 M HCl was injected into each culture to release dissolved CO_2 into the headspace which was analyzed for CO_2 by the gas chromatography (GC) method described previously (Chapter 2; Bressler *et al.* 1999).

4.2.6 Analytical methods

HFBT in aqueous samples was analyzed by HPLC using a Hewlett Packard model 1050 chromatography system and a 125 mm x 4 mm (5 μm) LiChrospher 100 RP-18 column (Hewlett Packard) with a UV detector at 394 nm. The mobile phase of acetonitrile:0.01 M K_2HPO_4 buffer, pH 9 (15:85) with a flow rate of 1 mL/min. The retention time for HFBT was 3.5 min.

A culture supernatant was analyzed for thioindigo using the same HPLC column with an acetonitrile:water (70:30) mobile phase flowing at 3 mL/min and the detector wavelength at 536 nm. The retention time for thioindigo (TCI America, Portland, OR) was 2.8 min.

Organic extracts containing HFBT or other sulfur heterocycles were analyzed by GC with a sulfur-selective detector (Chapter 2; Bressler *et al.* 1999). Some extracts were analyzed by low resolution GC-MS using a Hewlett Packard 5890 series II GC with a 5970 series mass selective detector and a 30-m DB-5 capillary column (J&W Scientific, Folsom, CA). High resolution GC-MS was done as outlined previously (Fedorak and Westlake 1986). The GC temperature program used for all of these analyses was 90°C of 1 min, 5°/min to 280°C for 21 min.

The molar extinction coefficient of HFBT in water at 394 nm was determined at three concentrations (84 μM, 56 μM and 28 μM) using a Unicam UV3 spectrophotometer (ATI Unicam).

4.3 RESULTS

4.3.1 Chemical Synthesis of HFBT

The chemical synthesis of HFBT gave unsatisfactory results because HFBT was only a minor product. A red product was formed from HFBT, through the action of sulfuric acid in the reaction mixture. This was the major product and HFBT could not be separated from it. Thus, the use of the chemical synthesis method was abandoned.

4.3.2 HFBT from culture extracts

The method for the production of HFBT from DBT by *Pseudomonas* strain BT1d was not optimized. At the end of the 2-months incubation period, the cultures were a dark reddish purple and there were always crystals of unused DBT in the culture flasks. After sublimation, the maximum HFBT yield from a 1.5-L culture was 80 mg.

The freshly collected yellow crystals had a melting point of 106-107°C, which matched the literature value of 107°C (Smiles and McClelland 1921; Yamada *et al.* 1968). GC analysis showed the purity to be greater than 99%. HPLC analysis showed only one peak. Figure 4.3 shows the mass spectrum of HFBT. The UV spectrum of HFBT in water showed a strong absorption at 394 nm, and the molar extinction coefficient was determined to be 12400 L/mol·cm.

Steam distillation was used in an attempt to improve the recovery of HFBT. However, during this procedure the HFBT appeared to undergo a reaction inside the condenser leaving a pink material coating the condenser walls. The addition of buffer at pH 7 to the collection flask stabilized the collected HFBT in an aqueous solution. Knowing the molar extinction coefficient of HFBT, desired amounts of HFBT from the collected condensate could be dispensed into cultures by adding the correct volumes of this aqueous solution. However, in most cases, the crystals collected by sublimation were used for these studies.

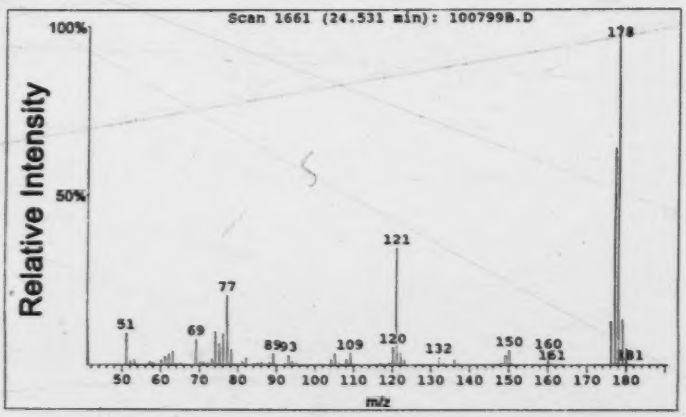


Figure 4.3 Mass spectrum of HFBT.

4.3.3 Abiotic transformations of HFBT in crystalline form

When HFBT was stored as crystals in sealed vials, a reddish purple color appeared on the crystals. This change also occurred when the crystals were stored under a N_2 atmosphere, in the dark, and at -20°C, but it was accelerated by the presence of O_2. Crystals that had been exposed to air for 1 year were examined to determine the product(s) of this reaction. TLC revealed at least four distinct colored compounds, one of which was HFBT.

Two dark pink bands were observed with R_f values of 0.82 and 0.75 when developed in the mixture of hexane and ethyl acetate. GC-MS analysis of the compound with an R_f of 0.75 showed a strong molecular ion at m/z 296 (with a relative intensity of 100%), with weak ions at m/z 298 (11), 297 (21) 240 (14), 120 (10), and 76 (8) (Figure 4.4a). This compound gave the same GC retention time (43.8 min) and mass spectrum as authentic thioindigo (Figure 4.4b).

The GC retention time of the other pink compound, with the R_f of 0.82, was 40.2 min. Its mass spectrum also gave a molecular ion of m/z 296, but a markedly different fragmentation pattern than thioindigo. The most abundant ions and their relative intensities (%) were 298 (15), 297 (26), 296 (100), 295 (70), 268 (94), 240 (76), 236 (16), 208 (14), 195 (17), 164 (13), 120 (24) (Figure 4.4c). The abundant fragments at m/z 268 and 240 suggested two sequential losses of C=O (M^+-28 and M^+-56, respectively). The Fourier transformed infrared spectrum of this compound revealed a strong absorption at 1716 cm^{-1} and a weaker absorption at 1283 cm^{-1}. These are characteristic of C=O stretching and bending vibrations in ketones. These results were consistent with this compound being *cis*-thioindigo. Figure 4.5 shows the structures of thioindigo and *cis*-thioindigo.

A purple compound remained at the origin of the TLC plate when the hexane-ethyl acetate solvent was used. With a more polar solvent system, the purple compound had an R_f of 0.69. After recovering this compound from the silica gel with methanol, attempts at GC-MS analysis and by probe electron impact ionization mass spectrometry were unsuccessful because of low volatility of the compound. Therefore, the identity of this compound remains unknown.

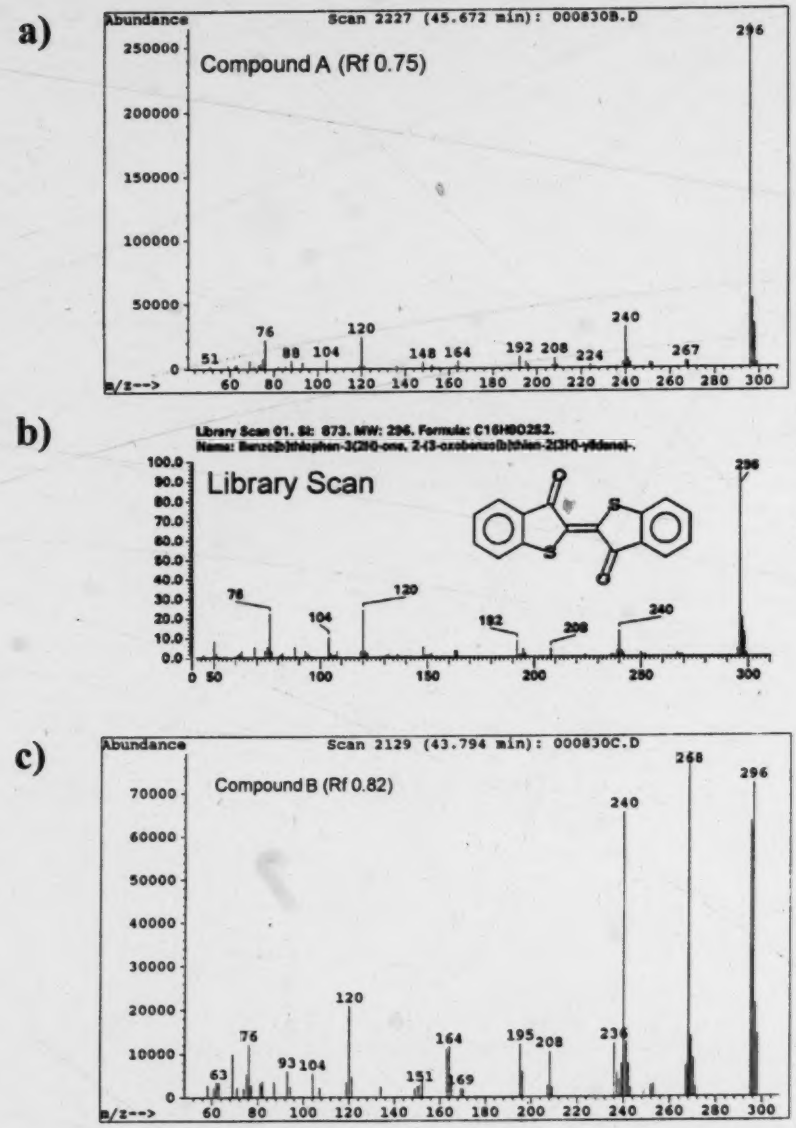
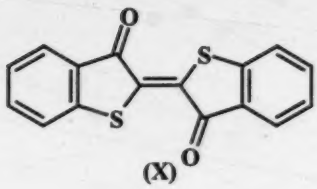
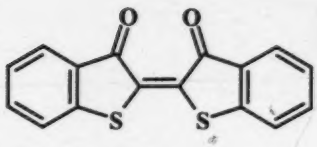


Figure 4.4 Mass spectrum of two compounds identified as products of abiotic HFBT condensation reactions. (a) Mass spectrum of unidentified product A with Rf 0.75 (b) Library scan acquired from the MASPEC II[32] Data System, Mass Spectrometry Services Ltd. Manchester, U.K. (c) Mass spectrum of unidentified product B with an Rf of 0.82.



(X)



(XI)

Figure 4.5 Structures of the pink products found with HFBT crystals after 1 year storage: (X) thioindigo and (XI) *cis*-thioindigo.

When purified HFBT was dissolved into organic solvents such as dichloromethane or ethyl acetate, the loss of HFBT became much more rapid than when the HFBT was stored as crystals. In solutions of dichloromethane, close to 10% of the HFBT was lost after only 5 days. Thus, HFBT was produced and purified in small batches, and stored as crystals.

4.3.4 Thioindigo in the supernatant of strain BT1d grown on HFBT

When thioindigo and *cis*-thioindigo were identified as abiotic reaction products from HFBT, efforts were made to detect these compounds in cultures of strain BT1d that were used to produce HFBT. After a neutral extraction with dichloromethane and concentration of the solvent, GC-MS analysis showed the presence of thioindigo (retention time 43.8 min), but not *cis*-thioindigo (retention time 40.2 min). HFBT, DBT sulfoxide, DBT sulfone, and residual DBT were also found in this extract (Figure 4.6).

It was possible that the thioindigo detected in the dichloromethane extract actually formed in the organic solvent, rather than in the culture of strain BT1d. Thus, an HPLC method was used for the direct analysis of the culture supernatant for thioindigo. This analysis showed the presence of a compound with an absorbance at 536 nm and the same HPLC retention time as authentic thioindigo. Therefore, the culture supernatant did contain thioindigo.

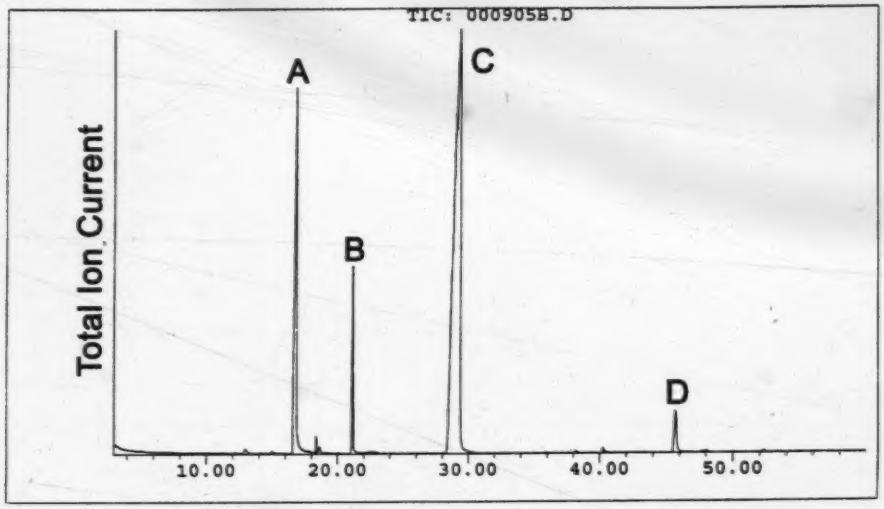


Figure 4.6 Chromatogram of the dichloromethane extract of a neutral culture supernatant of strain BT1d degrading DBT (B). The products are HFBT (A), co-eluting DBT sulfone and DBT sulfoxide (C) and thioindigo (D).

4.3.5 Stability of HFBT in sterile aqueous solutions

Freshly prepared aqueous solutions of HFBT were bright yellow, but these became a much lighter yellow color over time. In preparation for biodegradation studies, experiments were conducted to determine under which conditions HFBT was stable in sterile aqueous solutions in shake flasks. Figure 4.7 summarizes the results from the eight different conditions that were tested. The abiotic loss of HFBT occurred most rapidly in the shake flask that was exposed to light at pH 7, with essentially all of the HFBT gone by day 19. The HFBT was most stable at pH 11, whether in dilute NaOH or sulfate-free medium. At pH 11, less than 5% loss occurred over 19 days, and about 50% loss was observed over 55 days. At pH 7, HBFT was more stable in the sulfate-free medium than in the Milli-Q water (Figure 4.7). Over 19 days incubation, HFBT was essentially as stable in the sodium sulfide-reduced medium at pH 7, under a N_2 atmosphere, as it was in the aerated sulfate-free medium at pH 7. However, after 55 days, the HFBT was completely depleted in the latter medium, but about 30% remained in the reduced medium.

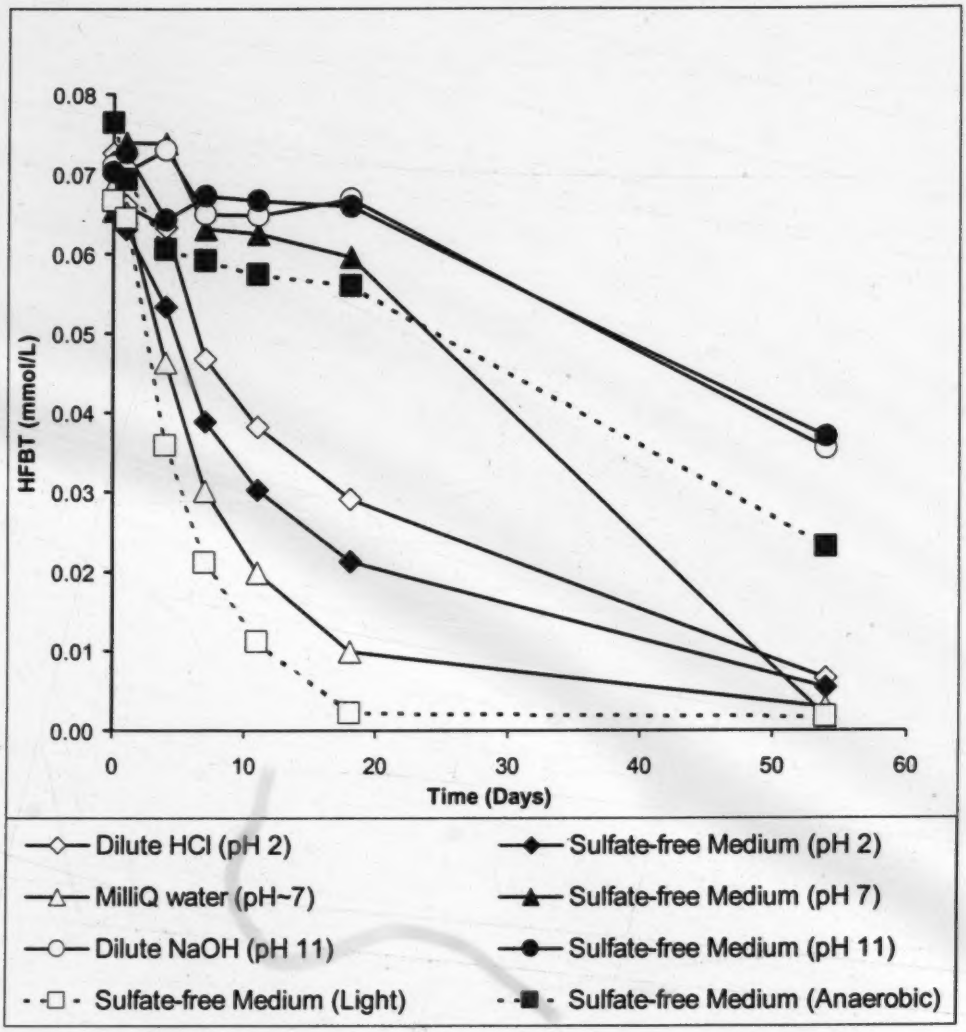


Figure 4.7 Stability of HFBT in various aqueous solutions at 28°C. For all but two cases, all the shake flasks were incubated aerobically in the dark. In one case the solution was incubated aerobically with illumination, and in the other case, the solution was incubated in the dark under a N_2 atmosphere after being reduced with sodium sulfide.

4.3.6 Biodegradation and mineralization of HFBT

During the enrichment of HFBT degraders from the SLPB mixed culture, portions of the cultures were transferred at 3-week intervals. According to the data in Figure 4.7, little abiotic loss of HFBT would be expected in mineral medium at pH 7 during this time. HPLC analyses showed that after 20 days incubation, the concentration of HFBT in the viable culture was 16% of that in the sterile control. Figure 4.8 compares the UV-visible spectra of the supernatants from a viable enrichment culture and a sterile control after 1 month of incubation. The strong absorption of HFBT at 392 nm was essentially removed by the viable culture.

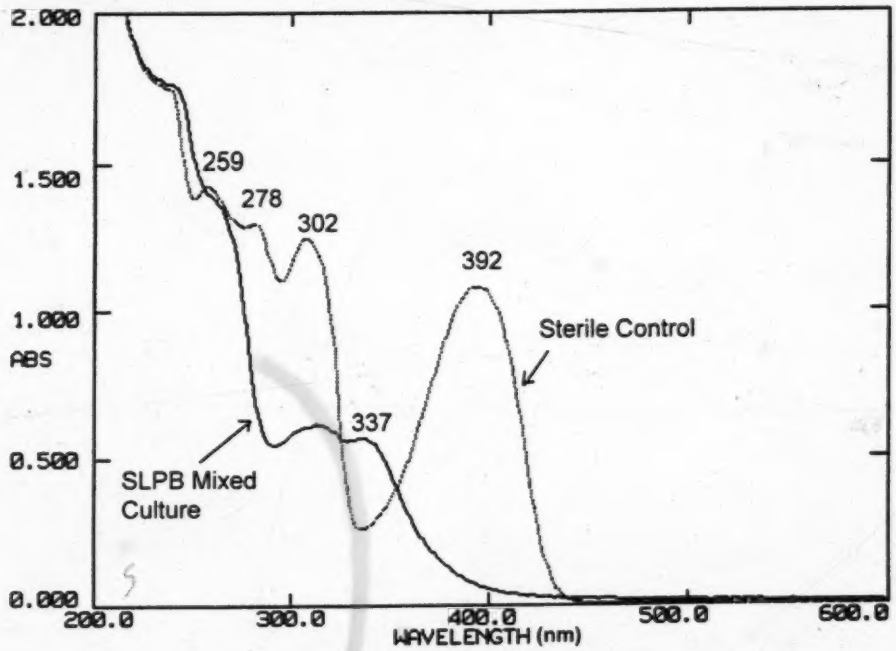


Figure 4.8 UV-visible spectra of the supernatant from a viable enrichment culture growing on HFBT for 1 month and a sterile control.

Benzothiophene-2,3-dione (VIII Figure 4.2) was often detected by GC-MS analysis of the dichloromethane extracts of acidified culture supernatants from SLPB cultures maintained on HFBT. Smaller amounts of the dione were detected in the corresponding sterile controls. This compound was never detected in the crystals of

HFBT collected by sublimation. At neutral pH, benzothiophene-2,3-dione exists as 2-mercaptophenylglyoxalate (VII Figure 4.2) (Eaton and Nitterauer 1994). Thus, the detection of the 2,3-dione in our culture extracts implies that 2-mercaptophenylglyoxalate was formed from HFBT in the sterile controls and to a greater extent in the viable cultures.

To determine if HFBT could be mineralized by the SLPB enrichment cultures, 0.039 mmol of this compound were added to each of three viable cultures incubated in sulfate-containing mineral medium in sealed serum bottles. After 14 days incubation, 50 ± 5.6% of the carbon from the HFBT was recovered as CO_2. In the heat-killed controls, the amount of CO_2 detected was equivalent to 6.9 ± 0.3% of the carbon added as HFBT. These results clearly demonstrated the mineralization of HFBT.

4.4 DISCUSSION

These investigations show that HFBT is a rather unstable intermediate of DBT biodegradation. In its crystal form and in dichloromethane solutions, it reacts to form thioindigo, *cis*-thioindigo, and a purple compound which could not be identified. HFBT is fairly stable at pH 11, and sufficiently stable in neutral sulfate-free medium (Figure 4.7) to allow culture studies over a 3-week incubation period.

The chemical synthesis method for HFBT was unsatisfactory, and Smiles and McClelland (1921) stated that the presence of mineral acids (H_2SO_4 was used during the synthesis) produced a red condensation product, with the same chemical structure as the keto form of compound (III) in Figure 4.1b. Kodama *et al.* (1970) described (III) as a violet compound that decomposed between 271~274°C, and based their identification on elemental analysis and on the work of Smiles and McClelland (1921). The purple compound that we detected as a contaminant of HFBT may have been (III) because it was not sufficiently volatile for GC-MS or probe mass spectrometry.

The microbiological method of producing HFBT yielded a mixture of metabolites from which HFBT was quite easily purified by sublimation. The maximum yield of 80 mg HFBT was only 4% of the theoretical yield from 2 g of DBT added to the culture. However, not all of the added DBT was consumed in the cultures, and there were other metabolites generated from DBT. Gas chromatography confirmed that the sulfoxide and

sulfone of DBT were produced by isolate BT1d. Kropp and Fedorak (1998) reported that 9% of the DBT added to cultures of BT1d was converted to the sulfoxide and/or sulfone of DBT that have been observed in the extracts of other bacterial cultures that degrade DBT via the Kodama pathway (Finkel'stein *et al.* 1997; Frassinetti *et al.* 1998; Kodama *et al.* 1970; Kropp *et al.* 1997a; Laborde and Gibson 1977).

Gas chromatography also revealed the presence of thioindigo in the neutral extract from the isolate BT1d. No studies that examined the biodegradation of dibenzothiophene demonstrating thioindigo formation could be found. In order to detect this compound, the GC temperature program had to be modified so that a higher final temperature of 280°C was reached. In previous work with DBT-degrading cultures (Kropp *et al.* 1997a), the final temperature was only 250°C, which presumably precluded the detection of thioindigo. I am not aware of any studies that have shown the biodegradation of thioindigo or its *cis* isomer.

In the analyses of the extracts of acidified culture supernatants from strain BT1d grown on DBT, there was no evidence of the presence of compounds IV (Figure 4.1b), V or VI (Figure 4.2) that were reported by other investigators. However, benzothiophene-2,3-dione (VIII) was frequently found, indicating the presence of 2-mercaptophenylglyoxalate (VII) in the neutral medium. Because the 2,3-dione was found in sterile controls, it appears that HFBT is abiotically transformed to 2-mercaptophenylglyoxalate in mineral medium. However, this process appears to be accelerated by microbial activity because the peak corresponding to the 2,3-dione in the GC analyses was always substantially larger than that in the sterile controls.

The biodegradation of 2-mercaptophenylglyoxalate (VII) has not been reported. Based on the numerous reports that have detected benzothiophene-2,3-dione and its methyl analogs in acidified culture extracts, these 2-mercaptophenylglyoxalates must be important intermediates in the biodegradation of benzothiophenes and dibenzothiophenes. For example, benzothiophene-2,3-dione has been found in cultures degrading benzothiophene (Bohonos *et al.* 1977; Eaton and Nitterauer 1994; Fedorak and Grbić-Galić 1991) and dibenzothiophene (Bohonos *et al.* 1977; Kropp *et al.* 1997a). In addition, methylbenzothiophene-2,3-diones have been detected in cultures degrading isomers of methylbenzothiophene (Kropp *et al* 1994b; Saftić *et al.* 1992), and isomers of

methyldibenzothiophene (Saftić *et al.* 1993) and 2,8-dimethyldibenzothiophene (Kropp *et al.* 1997b). Even dimethylbenzothiophene-2,3-diones have been observed in cultures degrading certain isomers of dimethylbenzothiophenes (Kropp *et al.* 1996) and 3,4-dimethyldibenzothiophene (Kropp *et al.* 1997b).

The study by Mormile and Atlas (1998) used a mixture of metabolites from DBT biodegradation and provided preliminary evidence that HFBT can be mineralized. However, my investigations using pure HFBT unequivocally demonstrated the release of CO_2 from HFBT. Subtracting the amount of CO_2 found in the sterile control, indicates that approximately 43% of the carbon in HFBT was released as CO_2. This is consistent with the proportion of carbon released from other sulfur heterocycles. In Chapter 2, it was observed that 57 and 62% of the carbon from benzothiophene sulfone and 3-methylbenzothiophene sulfone, respectively, was released as CO_2 by a *Pseudonocardia* sp. growing on these compounds. Three bacterial isolates that grow on sulfolane (tetrahydrothiophene-1,1-dioxide) liberated 40 to 42% of the substrate C as CO_2 (Greene *et al.* 2000).

Because of the limited supply of purified HFBT, I chose to use a medium that contained 7 mM sulfate for the growth of the HFBT-degrading mixed culture, rather than supplying HFBT as the sole sulfur source in sulfate-free medium. If all of the sulfur from HFBT had been released, this would have increased the total sulfate concentration by 0.49 mM. The high sulfate concentration in the medium precluded the detection of any sulfate that may have been formed by the release of the sulfur atom from HFBT. Other studies have shown that sulfur from thiophene rings can be used as the sole sulfur source for bacterial growth (Chapter 2; Bressler *et al.* 1999; Greene *et al.* 2000; van Afferden *et al.* 1990), so it is likely that HFBT would serve as a sole sulfur source. However, it is yet to be determined whether this is true.

There are three different modes of microbial attack on DBT [see Chapter 1 for review]. Two of these involve the initial oxidation of the sulfur atom yielding DBT sulfone, and the third, the Kodama pathway, begins with the oxidation of one of the homocyclic rings (Figure 4.1a). The so-called 4S pathway, leads to the selective removal of the sulfur atom, leaving a 2-hydroxybiphenyl which is not used as a carbon source by *Rhodococcus* strain IGTS8, the most extensively studied bacterium that carries out this

biodesulfurization. This mode of degradation occurs only in the absence of sulfate in the medium. Just one research group has reported the initial oxidation of the sulfur atom of DBT releasing sulfate followed by the cleavage of the homocyclic rings (van Afferden *et al.* 1990, 1993). *Brevibacterium* sp. DO is the only bacterium described that attacks DBT in this manner, leaving 9% of the carbon from dibenzothiophene as dissolved organic carbon in the medium (van Afferden *et al.* 1990). Based on the larger number of reports of bacteria producing HFBT (Bohonos *et al.* 1977; Frassinetti *et al.* 1998; Kodama *et al.* 1970, 1973; Laborde and Gibson 1977), it appears that in an environment that is not sulfate-limited, the Kodama pathway is likely the most common method for microbial degradation of DBT.

In addition, the Kodama pathway (Figure 4.1a) is commonly used by bacteria to degrade substituted DBTs, and the formation of methylHFBTs has been observed in cultures degrading methyDBTs and dimethylDBTs. For example, the degradation of each of the four isomers of methyl DBT yielded the corresponding methylHFBT (Saftić *et al.* 1993). Lu *et al.* (1999) demonstrated that 4,6-dimethylDBT could also be biodegraded to produce 7-methylHFBT, and Kropp *et al.* (1997b) observed that 3,4-dimethylDBT yielded 6,7-dimethylHFBT.

Thus, a better understanding of the biodegradation of HFBT, will lead to an understanding of the biodegradation of the methylHFBTs produced from methyl-substituted DBTs. The finding that benzothiophene-2,3-dione (arising from 2-mercaptophenylglyoxalate) originates from HFBT, suggests that the methyl-substituted benzothiophene-2,3-diones observed in culture extracts likely arise from methyl-substituted HFBTs.

Overall, these results suggest that the accumulation of HFBT in the environment would not likely occur because it is chemically quite unstable and it is susceptible to mineralization. Now that it has clearly been shown that HFBT can be biodegraded and mineralized by mixed bacterial cultures, it can be hypothesized that the methylHFBTs may also be biodegraded and mineralized. The testing of this hypothesis awaits the purification of some methylHFBTs to use as substrates for microbial cultures.

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5. ABIOTIC REACTION PRODUCTS FROM BENZOTHIOPHENE-2,3-DIONE AND THE BIODEGRADATION OF ONE OF THE PRODUCTS

5.1 INTRODUCTION

Sulfur is the third most abundant element in crude oils (Speight, 1980), and sulfur heterocycles are common constituents of petroleum and liquids derived from coal. Condensed thiophenes such as benzothiophene, alkylbenzothiophenes, dibenzothiophene and alkyldibenzothiophenes are amongst the most commonly found sulfur heterocycles. Based on data from Bence et al. (1996), it is estimated that during the *Exxon Valdez* spill, over 77 metric tonnes of dibenzothiophene and methyldibenzothiophenes were released into the environment. Previously, many research groups have investigated the microbial biodegradation of condensed thiophenes such as benzothiophene and dibenzothiophene as well as alkylbenzothiophenes and dibenzothiophenes (Atlas *et al.* 1981; Bohonos *et al.* 1977; Eaton and Nitterauer 1994; Fedorak and Grbić-Galić 1991; Fedorak and Westlake 1982, 1984; Hostettler and Kvenvolden 1994; Kargi and Robinson 1984; Kodama *et al.* 1970, 1973; Kropp *et al.* 1994a, 1997a; Laborde and Gibson 1977; Monticello *et al.* 1985; Saftić *et al.* 1992, 1993; van Afferden *et al.* 1990). The alkyl condensed thiophenes are among the most recalcitrant compounds in petroleum-contaminated environments (Boehm *et al.* 1981; Bence *et al.* 1996) although methyl and dimethyldibenzothiophenes have been shown to be susceptible to biodegradation (Fedorak and Westlake 1982, 1984; Kropp *et al.* 1997a).

Often when contaminating compounds are biodegraded, they are oxidized to more polar metabolites (Bragg *et al.* 1994). It is important to identify as many polar metabolites as possible in order to assess the toxicity and carcinogenic nature of these compounds. Based on the Microtox assay, Zemanek (1994) was able to demonstrate that in several cases, the polar fraction from petroleum-contaminated sites was the most toxic fraction of the residual petroleum. Bragg *et al.* (1994) recognized that during the biodegradation of oil released from the *Exxon Valdez* spill, the polar content of the biodegraded North Slope oil approached 60-70% of the total mass, and the biodegradation slowed substantially. Therefore, it is important to follow the metabolism

of compounds such as dibenzothiophene, and determine complete degradation pathways, in order to determine which polar metabolites are produced during degradation.

Previous studies have shown that 3-hydroxy-2-formylbenzothiophene (HFBT) is a metabolite of dibenzothiophene that accumulates in pure cultures (Kodama *et al.* 1970). Work by Mormile and Atlas (1988) suggested that HFBT can be further biodegraded, but did not reveal anything about the fate of the carbon and sulfur atoms. Chapter 4 (and Bressler and Fedorak 2001) revealed many chemical properties of purified HFBT and described the abiotic condensation of HFBT to form *cis*- and *trans*-thioindigo. Chapter 4 also described the identification of benzothiophene-2,3-dione as a biodegradation product of HFBT and showed that HFBT could be mineralized by a mixed bacterial population.

Bohonos *et al.* (1977) found benzothiophene-2,3-dione in culture extracts from dibenzothiophene grown cultures and suggested it to be an oxidized metabolite in the HFBT producing pathway. This is supported by the findings of Chapter 4 (Bressler and Fedorak 2001). The 2,3-dione has also been identified as a dibenzothiophene metabolite in HFBT-producing cultures (Kropp *et al.* 1996) and is thought to be formed from the cyclization of 2-mercaptophenylglyoxalate (Eaton and Nitterauer 1994) (Figure 5.1). Finkel'stein *et al.* (1997) have identified mercaptobenzoic acid, (or thiosalicylic acid) as a further metabolite of dibenzothiophene degradation. To date, there is no proof that mercaptobenzoic acid is formed directly from the 2,3-dione and not from a concurrent metabolic pathway.

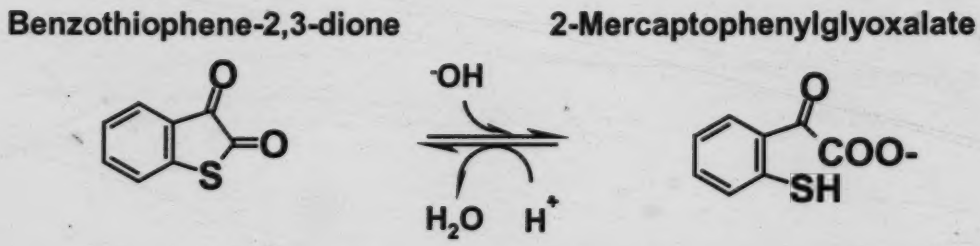

Figure 5.1. Equilibrium between benzothiophene-2,3-dione and 2-mercaptophenyl-glyoxalate.

Benzothiophene-2,3-dione, or alternatively 2-mercaptophenylglyoxylate, also appears to be a biodegradation metabolite of benzothiophene. Eaton and Nitterauer (1994) reported benzothiophene biotransformation by isopropylbenzene-degrading bacteria. One of the metabolites identified was 2-mercaptophenylglyoxalate which existed when the culture was at neutral pH, but at acidic pH used for culture extraction to obtain metabolites, the compound cyclized to form benzothiophene-2,3-dione (Figure 5.1). The 2,3-dione has also been observed in the extracts of acidified cultures incubated with methyl substituted benzothiophenes containing the alkyl substituent on the benzene ring (Fedorak and Grbić-Galić 1991; Saftić *et al.* 1992; Kropp *et al.* 1994a). Andersson and Bobinger (1992) reported that when benzothiophene is subjected to photooxidation, it is converted through benzothiophene-2,3-dione to 2-sulfobenzoic acid with a 85% yield of the acid. No investigations into microbial attack of the 2,3-dione have been reported.

2,2'-Dithiosalicylic acid was identified as a product of dibenzothiophene biodegradation by Finkel'stein *et al.* (1997). They suggested that 2,2'-dithiosalicylic acid is actually a product of mercaptobenzoic acid oxidation forming the disulfide. Other than 2,2'-dithiosalicylic acid, no other disulfides have been reported as products of dibenzothiophene biodegradation. Recent literature searches have revealed no investigation on the biodegradation or environmental fate of 2,2'-dithiosalicylic acid.

The first objective of this study was to determine many of the chemical properties of benzothiophene-2,3-dione, specifically abiotic reactions of the dione when dissolved in an aqueous phase at various pH values. The goal was to understand if benzothiophene sulfone, or 2-mercaptophenylglyoxylate, would be stable enough at neutral pH to allow further biodegradation studies. A second objective was to reveal information on the biodegradation of 2,2'-dithiosalicylic acid, an identified abiotic product of 2-mercapto-phenylglyoxylate condensation and decarbonylation.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

Dibenzothiophene (>98%) was purchased from Fluka (Buch, Switzerland). Acetonitrile and dichloromethane (HPLC grade) were from Fisher Chemicals (Fair Lawn, N.J.). Diazald, thiophenol, and oxalyl chloride were obtained from Aldrich (Milwaukee,

WI). 2,2'-Dithiosalicylic acid (95% purity) was obtained from Lancaster Synthesis (Windham, NH). Diethylether was obtained from BDH Inc. (Toronto, ON).

5.2.2 Chemical synthesis of benzothiophene-2,3-dione

Benzothiophene-2,3-dione was synthesized by the method of Hannoun *et al.* (1982). Oxalyl chloride in ether was added dropwise to an ethereal solution of thiophenol and then refluxed. After removal of the ether and desiccation over potassium hydroxide pellets, the product was treated with aluminum chloride and then decomposed with HCl. Crude benzothiophene-2,3-dione produced through this synthesis was further purified by two recrystalizations from methanol. The large bright orange crystals were found to have a melting point of 119-120°C which agreed closely with values obtained by Seymour et al. of 117-120°C (1997). The crystals were found to have greater than 99% purity by high performance liquid chromatography (HPLC) and by gas chromatography analysis (GC).

5.2.3 Abiotic reactions of benzothiophene-2,3-dione at various pH

Benzothiophene-2,3-dione was dissolved in 300 mL of B+N8P, a strongly phosphate buffered mineral salts medium, previously described by Kropp *et al.* (1994a). The dione was dissolved at a concentration of 100 mg/L in 500 mL Erlenmeyer flasks and the medium was adjusted to pH 1, 3, 5, 7, 9 and 11 with HCl or NaOH. The solutions were then filter sterilized and transferred into sterile Erlenmeyer flasks and incubated in the dark with shaking at 200 rpm. Sampling was accomplished by aseptically withdrawing 1 mL samples which were then subjected to HPLC and pH analysis. After a 1 month incubation, all contents of the flasks were acidified with 5 mL of concentrated HCl to pH < 1 and then extracted with dichloromethane. The extract was treated with concentrated diazomethane (generated from Diazald according to the manufacturers instructions), and then subjected to GC with a mass selective detector (GC-MS) analysis.

5.2.4 Detection and identification of disulfides formed abiotically from benzothiophene-2,3-dione

Abiotic incubations of benzothiophene-2,3-dione were conducted in sterile sulfate-free mineral salts medium, (Chapter 2; Bressler *et al.* 1999), at neutral pH. Twenty milligrams of the dione were incubated in 200 mL of sulfate-free medium in 500-mL Erlenmeyer flasks. After 20 days incubation, the solutions were acidified with 5 mL of concentrated HCl resulting in a pH <1. The solutions were then extracted three times with 50 mL of dichloromethane and the pooled extracts were evaporated under reduced pressure to 1 mL. The concentrated extract was then transferred to a 1 dram vial and taken to dryness under a nitrogen stream. The resulting residue was then derivatized with an ethereal diazomethane solution. The derivatization was allowed to continue for 1 h and then the sample was again evaporated to dryness with a nitrogen stream. The sample was redissolved in 100 µL of dichloromethane and subjected to GC-MS analysis. Production of products from the 2,3-dione was also monitored by HPLC.

5.2.5 Effect of temperature and light on benzothiophene-2,3-dione stability

To determine if temperature or exposure to bright light would affect disulfide formation or stability, sterile 200 mL shake flask solutions with 100 mg/L benzothiophene-2,3-dione in B+N8P medium were incubated for 18 days. The first set of triplicate solutions were incubated at 28°C wrapped in aluminum foil (28°C Dark), the second at 28°C constantly exposed to a 40 watt bulb at a distance of 15 cm (28°C Light), and the third set of flasks was incubated at 4°C wrapped in aluminum foil (4°C Dark). One milliliter samples were aseptically withdrawn at various times and analyzed as described above for the presence of the disulfides and loss of the 2,3-dione.

5.2.6 Mixed culture biodegradation of 2,2'-dithiosalicylic acid

Samples of two garden soils (10 grams of each) were inoculated into 200 mL of filter sterilized sulfate-free mineral salts medium which contained 50 mg/L 2,2'-dithiosalicylic acid in 500-mL Erlenmeyer flasks. The cultures were incubated in the dark with shaking at 200 rpm. Biodegradation of the disulfide was monitored by HPLC

and by pH decrease. Upon removal of the disulfide, after approximately 20 days, 10 mL of the culture was transferred to fresh medium. To follow removal of 2,2'-dithiosalicylic acid from the medium, samples were monitored by HPLC to determine the extent of substrate removal over time. As the cultures degraded the disulfide, the pH was also monitored to check for acid production.

To determine the extent of mineralization, carbon balance experiments were conducted with the mixed bacterial populations. The two garden soil cultures, after five enrichment transfers in 2,2'-dithiosalicylic acid containing sulfate-free mineral salts medium, were inoculated into sealed 160-mL serum bottles in triplicate. Ten milliliters of the cultures were inoculated into 80 mL of filter sterilized sulfate-free mineral salts medium containing 100 mg/L 2,2'-dithiosalicylic acid. Before sealing the serum bottles, 15 mL of the inoculated culture was removed acidified with 0.1 mL of concentrated HCl and analyzed for dissolved organic carbon, total organic carbon, and by HPLC for substrate concentration. The remaining 75 mL of culture was sealed into the serum bottle and injected with 10 mL of air creating positive air pressure. Equivalent filter sterilized controls were also included. The bottles were then incubated in the dark at 28°C for 6 weeks. After incubation, the bottles were acidified with 0.5 mL of concentrated HCl and analyzed for the same parameters as time zero samples, and for CO_2 production.

5.2.7 Isolation of 2,2'-dithiosalicylic acid degraders

The two garden soil mixed cultures were subjected to five rounds of enrichment transfers. The cultures were then streaked for isolated colonies on Plate Count Agar (PCA, Difco Laboratories, Detroit, MI) and incubated in the dark at 28°C. Four distinctive colony types were observed and each was restreaked to obtain isolated bacterial cultures. These four bacterial isolates were then inoculated individually into 2,2'-dithiosalicylic acid containing sulfate-free mineral salts medium to test for biodegradation ability.

5.2.8 Identification of disulfides as dibenzothiophene biodegradation products

Pseudomonas BT1d cultures were used previously (Chapter 4; Bressler and Fedorak 2001) to produce HFBT from dibenzothiophene as the sole carbon and energy source. This isolate was inoculated into several 2-L Erlenmeyer flasks that each contained 1.5 L of B+N8P mineral salts medium and 2 g of dibenzothiophene (most of which remained as crystals). These cultures were incubated at 28°C with shaking for 2 months. After incubation, 500 mL of this culture was acidified with 10 mL of concentrated HCl and extracted with three 100-mL portions of dichloromethane. The extracts were pooled, concentrated and derivatized with diazomethane. The resulting derivatized mixture was then subjected to GC-MS analysis.

5.2.9 Quantitative determination of sulfur as high molecular weight biodegradation metabolites

In order to determine the amount of dibenzothiophene sulfur converted to large molecular weight disulfides and thioindigo upon biodegradation by various *Pseudomonas* strains, mass balance experiments were conducted. Four *Pseudomonas* strains, including BT1d, BT1, W1 and F, (all previously described by Kropp and Fedorak 1998) were inoculated in triplicate into 200 mL B+N8P mineral salts medium cultures containing 20 mg/L dibenzothiophene as well as 50 µL of 1-methylnaphthalene (all except BT1d cultures) as described by Kropp and Fedorak (1998). After 11 days incubation, the cultures underwent three successive organic extractions. The first extraction involved a neutral extraction at pH 7 after addition of 4-methyldibenzothiophene as an internal standard. The cultures were extracted three times with 50 mL of dichloromethane to remove residual dibenzothiophene, thioindigo and other neutrally charged compounds.

The subsequent round of extraction involved the addition of 3-methylbenzothiophene sulfone as an internal standard, an acidification to pH 3 using citric acid (Chapter 4; Bressler and Fedorak 2001), and three extractions with 50 mL of dichloromethane to remove all HFBT and benzothiophene-2,3-dione. The third extraction involved addition of thianthrene as the internal standard, further acidification with 5 mL of concentrated HCl to pH <1, and three 50 mL extractions with dichloromethane to extract any disulfides formed. All extracts were concentrated under reduced pressure in a rotovap and subsequently under a nitrogen stream to approximately

100 µL. The third extracts (pH<1) were then concentrated to dryness, derivatized with diazomethane, and then redissolved in 100 µL dichloromethane. All extracts were analyzed by GC-MS and then selected extracts were analyzed by GC with an atomic emissions detector monitoring sulfur concentrations.

5.2.10 Analytical methods

Benzothiophene-2,3-dione in aqueous samples was analyzed by HPLC using a Hewlett Packard model 1050 chromatography system and a 125 mm x 4 mm (5 µm) LiChrospher 100 RP-18 column (Hewlett Packard) with a UV detector at 240 nm. The flow rate was 2 mL/min. In order to properly quantify the 2,3-dione, the decision was made to use an acidic (pH~1) high salt HPLC mobile phase using phosphoric acid (1.5%), phosphate buffer (64%, 0.01M), and acetonitrile (34.5%).

This acidic high salt buffer had the advantage of forcing the 2,3-dione equilibrium in favor of the 2,3-dione allowing separation of the 2,3-dione from the void volume during HPLC analysis. The retention time for the 2,3-dione was around 3.5 min. Subsequent experiments using HPLC analysis demonstrated that over time, the 2,3-dione to 2-mercaptophenylglyoxylate equilibrium was interrupted by the loss of the 2-mercaptophenylglyoxylate to other observable unknown compounds. To confirm the identity of the 2-mercaptophenylglyoxylate and to help characterize the unknown peaks that formed over time, the acidic mobile phase and RP-18 HPLC column were transferred to a Waters 2690 HPLC system which was equipped with a Waters 996 photodiode array detector allowing UV-Vis scans for each eluted peak.

An Agilent Technologies 1100 MSD was used for low resolution HPLC-MS. In order for HPLC to be used in conjunction with mass spectrometry, there can be no inorganic salts present in the mobile phase. Gradient elution using an acetonitrile-water mobile phase, from 0 to 50% acetonitrile allowed the identification of the primary product formed when the 2,3-dione was lost.

To obtain a molecular formula of the primary product formed after 2,3-dione loss, a Micromass ZabSpec oaTOF instrument was used for direct loop injection electrospray high-resolution mass-spectrometry under both positive and negative ionization conditions.

Organic extracts containing benzothiophene-2,3-dione or other sulfur heterocycles were analyzed by GC with a sulfur-selective detector (Chapter 2; Bressler *et al.* 1999). Some extracts were analyzed by low resolution GC-MS using a Hewlett Packard 5890 series II GC with a 5970 series mass selective detector and a 30-m DB-5 capillary column (J&W Scientific, Folsom, CA). High resolution GC-MS was done as outlined previously (Fedorak and Westlake 1986). The GC temperature program used for all of these analyses was 90°C of 1 min, 5°/min to 280°C for 21 min.

To obtain total organic carbon and dissolved organic carbon concentrations in culture supernatants, a Shimatzu Model TOC-5000A Total Organic Carbon Analyzer attached to a Model ASI-5000A autosampler was used. Sulfate determinations were performed using a Dionex Series 2000 i/sp ion chromatograph with an Ion Pac AS4A-SC 4-mm I.D. column with a conductivity detector.

5.3 RESULTS

5.3.1 Abiotic benzothiophene-2,3-dione transformation products

Benzothiophene-2,3-dione is not stable at neutral or alkaline pH. Eaton and Nitterauer (1994) showed that the 2,3-dione undergoes a reversible ring opening reaction to form 2-mercaptophenylglyoxylate (Figure 5.1). During this investigation, it was observed that the 2,3-dione was chemically stable at low pH (<2) but at higher pH, 2-mercaptophenylglyoxylate tended to form and then rapidly disappear from aqueous solution.

To follow benzothiophene-2,3-dione loss over time, a HPLC mobile phase was developed which was highly acidic, and buffered strongly with phosphate. As illustrated in Figure 5.2, even with this highly acidic mobile phase, the 2,3-dione equilibrium could not be shifted completely to the closed ring form entirely. A significant proportion of the 2-mercaptophenylglyoxylate could be observed (Compound B, Figure 5.2) when a pH 11 mercaptophenylglyoxylate containing NaOH solution was added. This finding indicated that the ring closure equilibrium was not rapid and an increased baseline could be observed between the two peaks. It is believed that the increased baseline was caused by 2-mercaptophenylglyoxylate undergoing the ring closure reaction during the time of the

chromatographic run. This phenomenon was lessened by using the highly buffered mobile phase, but meant that quantitation by HPLC of the two peaks was impossible.

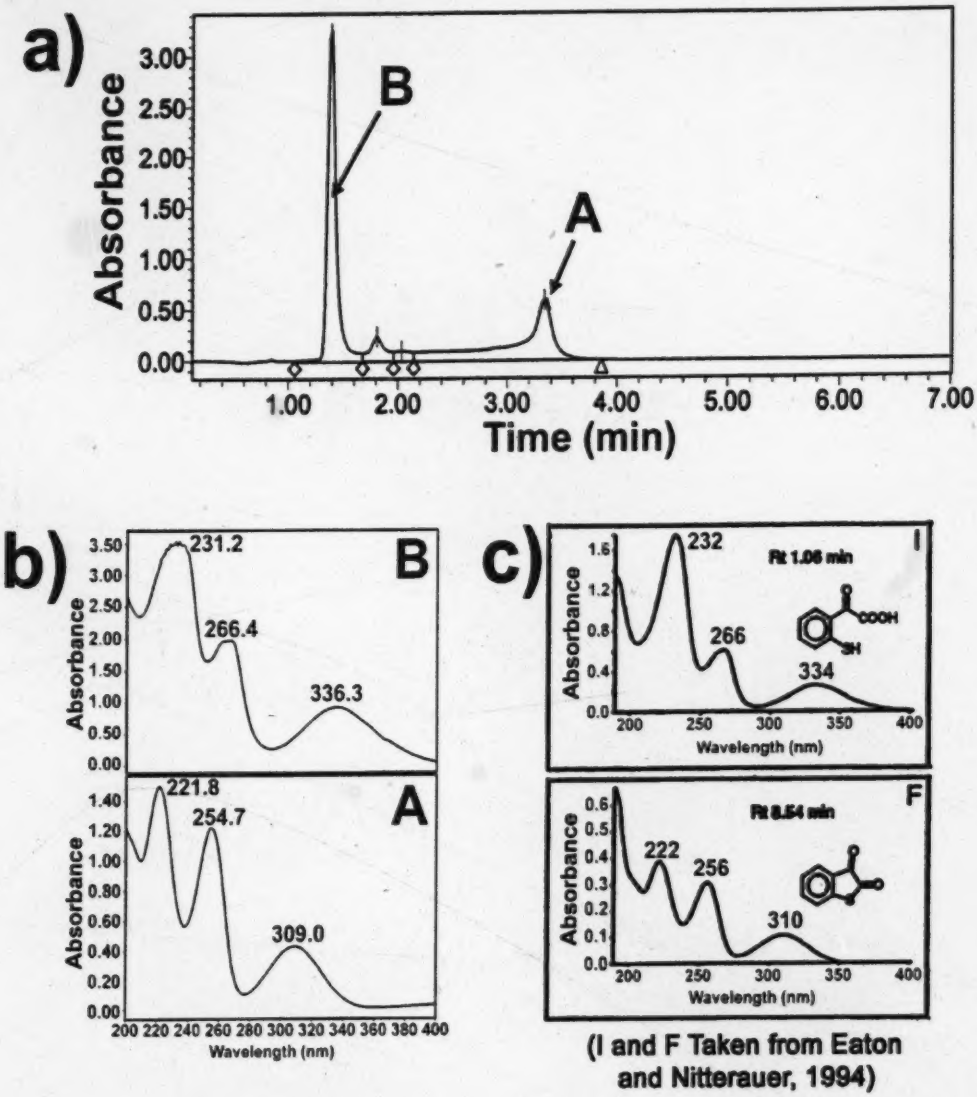

Figure 5.2 HPLC diode array analysis of benzothiophene-2,3-dione in a pH 11 NaOH solution. (a) HPLC chromatograph and (b) diode array spectrum scans compared to (c) literature scans.

When benzothiophene-2,3-dione, or more correctly 2-mercaptophenylglyoxylate, was incubated at pH 7 in mineral salts medium, the two peaks in the HPLC chromatograph (A and B in Figure 5.3) were observed to disappear rapidly. The disappearance of these two peaks coincided with the formation of a unknown peak (C) with almost complete conversion within 24 h. The newly formed peak (1.3 min retention time) was observed to transform slowly into a fourth compound (D in Figure 5.3) within hours and a fifth compound (not shown, retention time 4.9 min) within a few days.

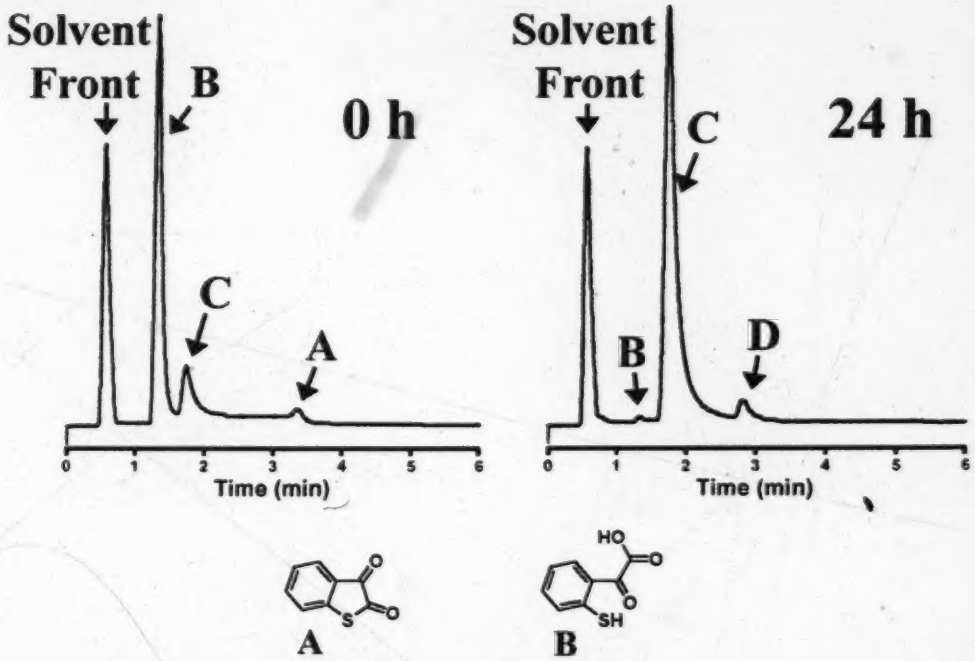

Figure 5.3 Benzothiophene-2,3-dione (100 mg/L) incubated for 24 h at pH 7 in sulfate-free mineral salts medium.

To identify these transformation products, a solution containing mainly the first product (compound C) was subjected to HPLC mass selective detection under both positive and negative ionization conditions. As illustrated in Figure 5.4, a variety of ions were observed. Further analysis of the spectra revealed that the positive spectra (Figure 5.4a) resembled a compound with a molecular ion at 400 (401 – 1H) mass units, with the addition of one potassium (m/z = 438.9) and two potassium (m/z = 476.9) ions. Further

analysis of the negative ionization mass spectrum (Figure 5.4b) revealed that the actual mass of the product was probably 362 (361 + 1H) with the addition of one potassium (400).

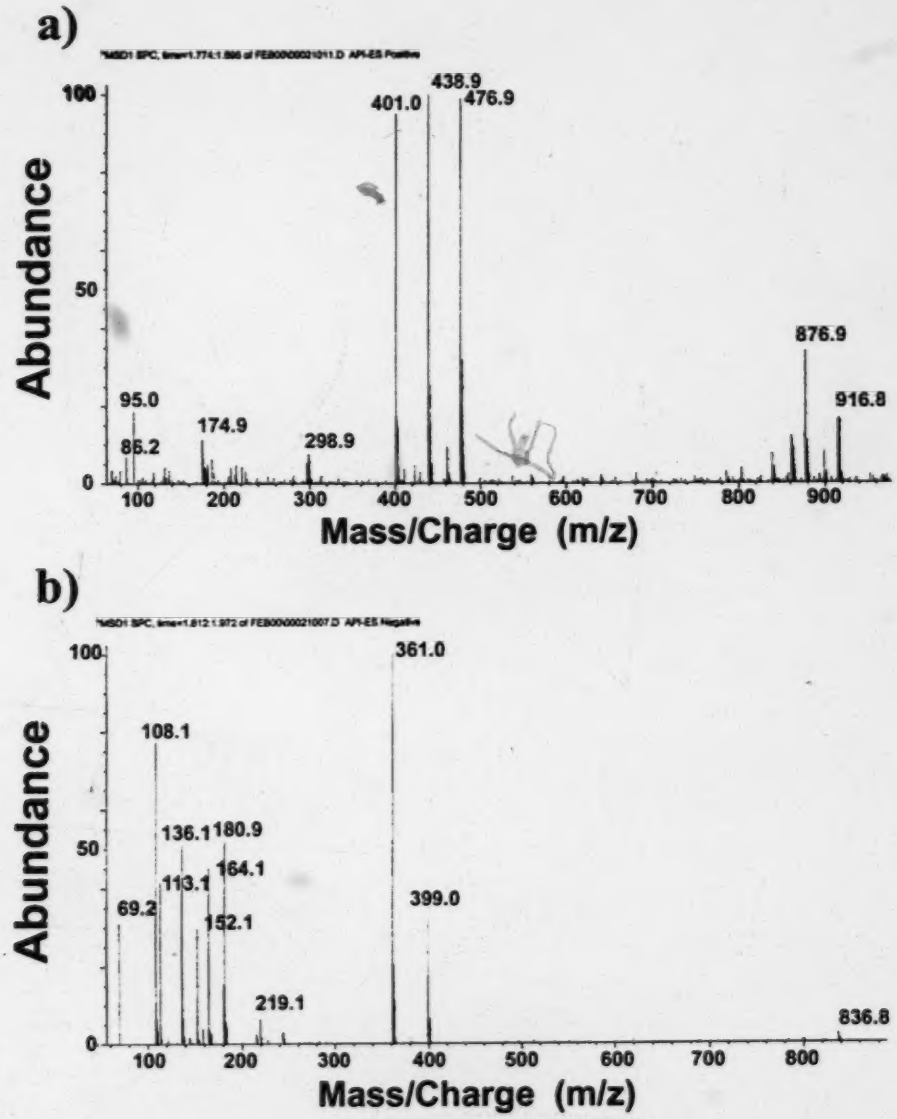

Figure 5.4 Positive (a) and negative (b) ionization mass spectra of product C (1.9 min retention time) obtained through HPLC with a mass selective detector.

The findings of the negative ionization mass spectrum suggested that the observed 401 molecular weight in the positive spectrum already contained 1 potassium ion. These findings were confirmed by high resolution mass spectrometry which revealed the actual molecular formula to be $C_{16}H_{10}O_6S_2K_1$ with a molecular weight of 400.955516. Other potassium adducts were identified with the formula $C_{16}H_{10}O_6S_2K_2$ (mass = 438.911678) and $C_{16}H_{10}O_6S_2K_3$ (mass = 476.868088). The potassium adducts are present due to the fact that the mineral salts medium used for the experiment was buffered with potassium. This molecular formula was consistent with the oxidation of 2-mercaptophenylglyoxylate resulting in the formation of a disulfide (Figure 5.5).

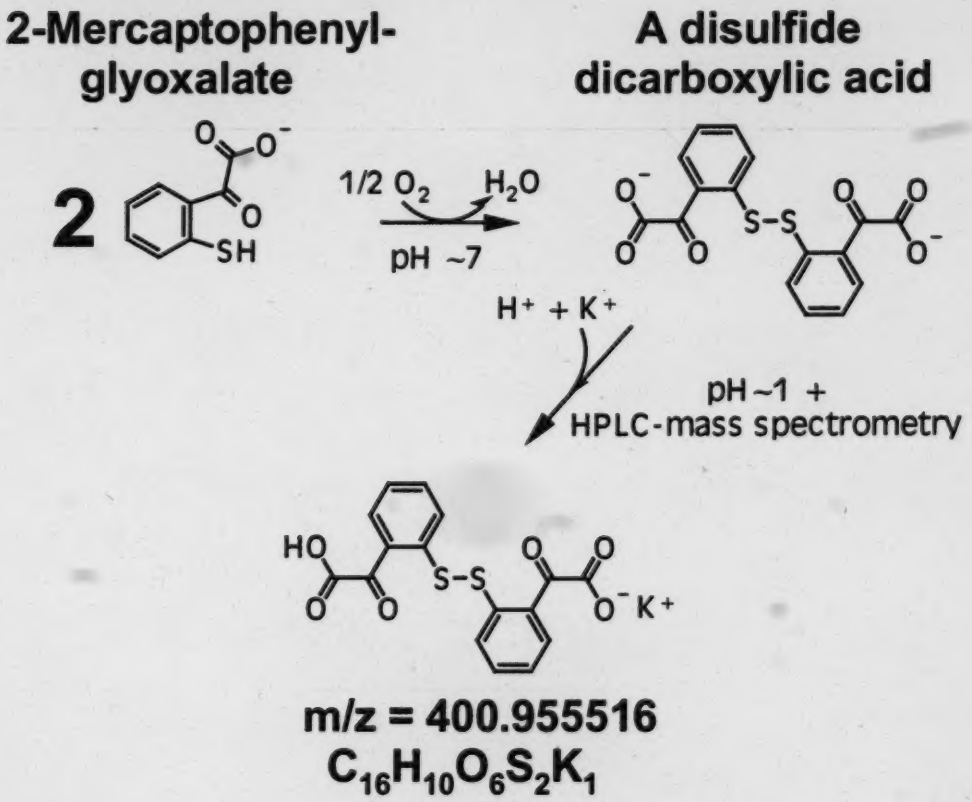

Figure 5.5 Oxidation of 2-mercaptophenylglyoxylate to a disulfide

It was decided to attempt GC-MS analysis of acidic (pH 2) dichloromethane extracts of sterile solutions incubated with benzothiophene-2,3-dione in mineral salts medium to identify the unknown products. Efforts using GC-MS protocols with an oven temperature up to 250°C failed to show any peaks other than benzothiophene-2,3-dione. It was observed that a faint yellow color was extracted if the solutions were extracted at pH <1. Subsequently, a diazomethane derivatization procedure was employed in combination with a hotter GC oven temperature profile. As illustrated in Figure 5.6a, new peaks were observed (peaks C, D and E).

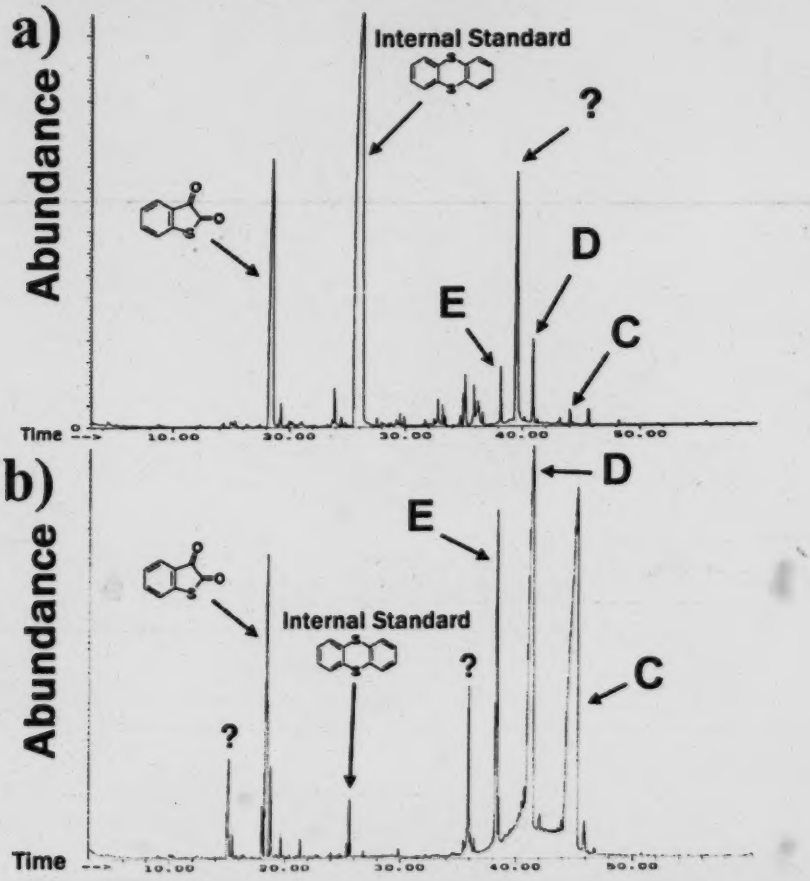

Figure 5.6 Anhydrous sodium sulfate dried acidified extract of benzothiophene-2,3-dione transformation products formed through the incubation of sterile benzothiophene-2,3-dione (100 mg/L) in sulfate-free mineral salts medium at neutral pH (a) Non-dried extract of redissolved sodium sulfate drying agent (b)

A second observation was that a considerable amount of material appeared to be precipitating during the acidification prior to extraction. An anhydrous sodium sulfate drying agent was previously used to dry extracts prior to GC-MS analysis. To confirm loss of material to the drying agent, the drying agent was redissolved in a 0.1 M HCl solution. A second dichloromethane extraction was performed and the resulting extract was concentrated to the same volume (1 mL) as the original extract, but not dried. After diazomethane derivatization, GC-MS analysis of this second extract (Figure 5.6b) demonstrated large quantities of three new products (compounds C,D, and E). It was apparent that these three products were relatively insoluble in both dichloromethane and water under acidic conditions. Subsequent experimentation revealed that only tetrahydrofuran served as a good solvent for these compounds.

As shown in Figure 5.7, all three products have apparently related spectra with fragments at either, or both 167 and 195. The molecular ions all differ by only 28 mass units (indicating a loss of 28 mass units between each product). As diagramed in Figure 5.7 the spectra of the largest product is consistent with the disulfide oxidation product of 2-mercaptophenylglyoxylate. The second product appears to have undergone a single decarbonylation reaction. The third product was observed to have a mass spectrum which suggested two decarbonylation reactions resulting in 2,2'-dithiosalicylic acid.

Commercially available 2,2'-dithiosalicylic acid was subjected to diazomethane derivatization and GC-MS analysis to help confirm the identification of the third product (Figure 5.8). 2,2'-Dithiosalicylic acid standard was also subjected to HPLC analysis with a diode array detector. By comparing a 1-month-old solution of benzothiophene-2,3-dione products (Figure 5.9b) to authentic standard (Figure 5.9a) the HPLC 4.9 min peak was confirmed to be 2,2'-dithiosalicylic acid. Figure 5.9c provides the UV-Vis scans for the other two disulfide products. Unfortunately authentic standards were not available for comparison.

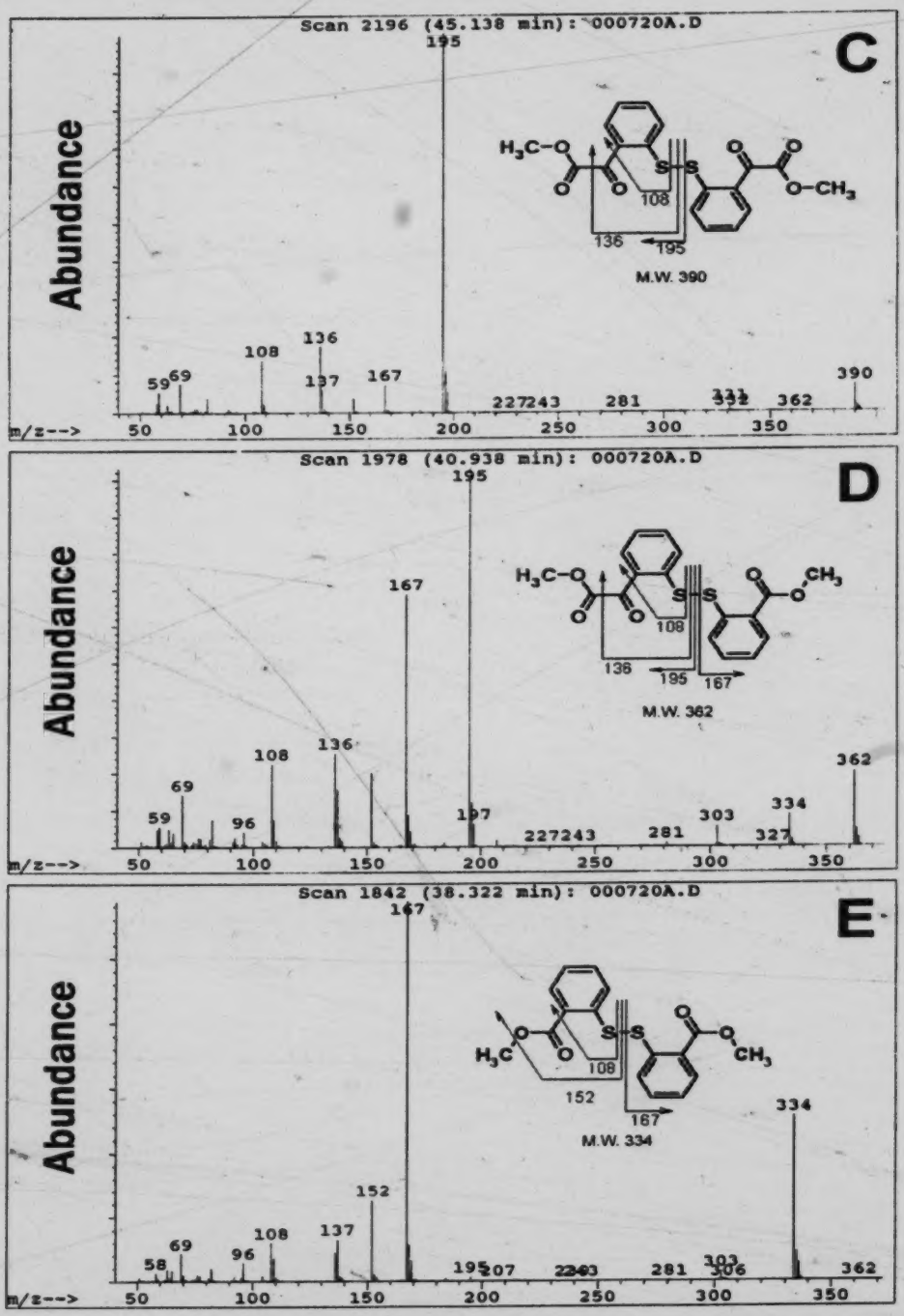

Figure 5.7 Mass spectrum of the three disulfides formed through 2-mercaptophenyl-glyoxalate oxidation and subsequent decarbonylation.

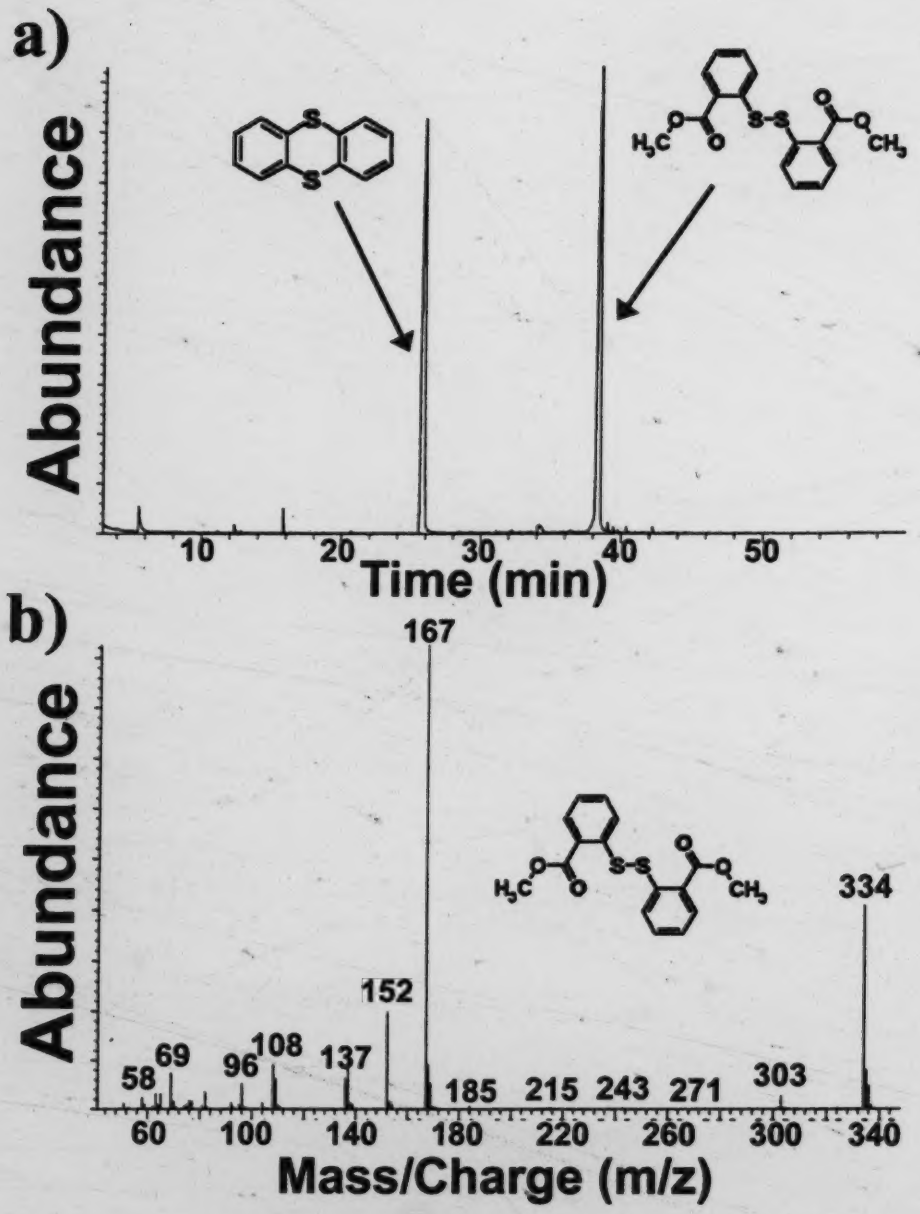

Figure 5.8 Total ion current of authentic 2,2'-dithiosalicylic acid (and thianthrene standard) derivatized with diazomethane (a) mass spectrum of the dimethyl ester of 2,2'-dithiosalicylic acid (b)

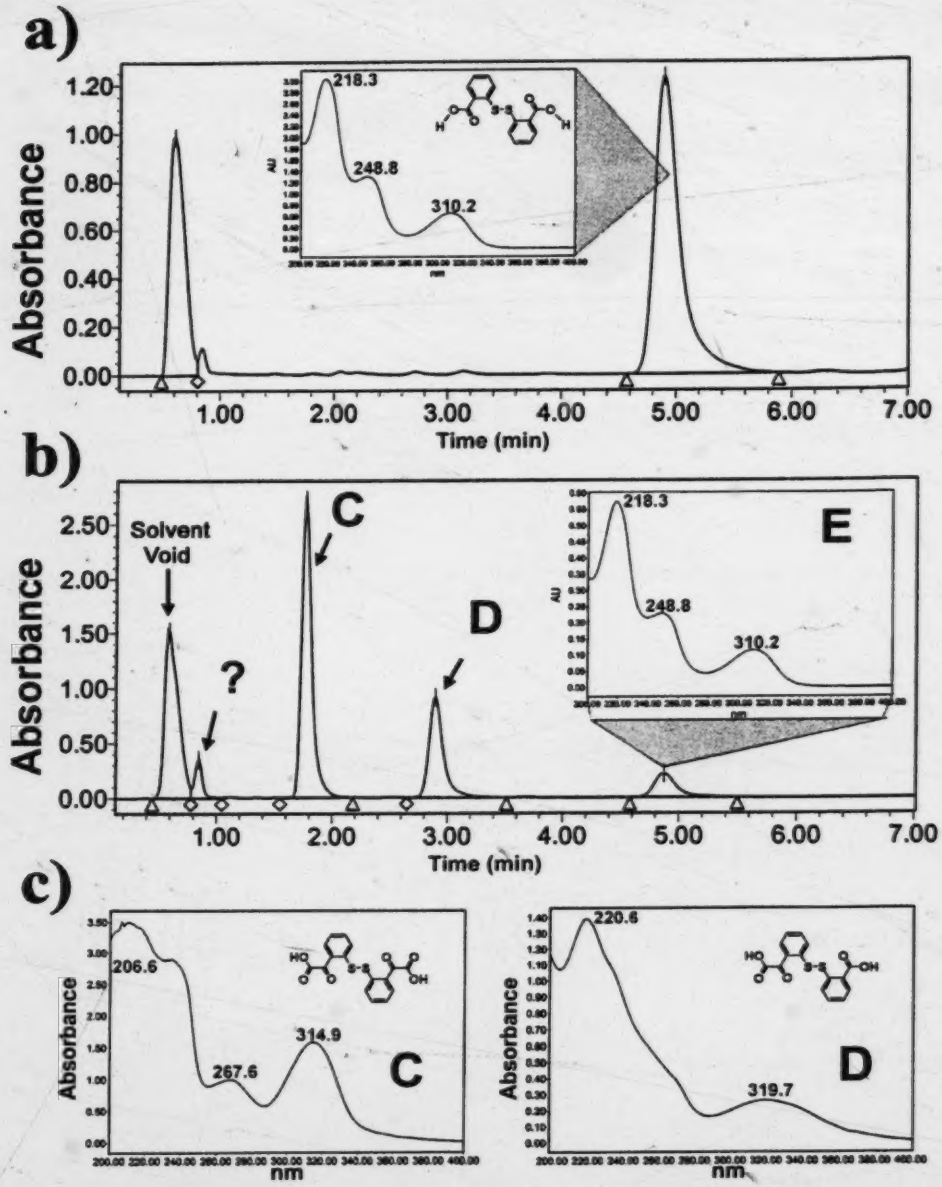

Figure 5.9 HPLC diode array analysis of a 1 month old pH 9 solution of disulfides formed through abiotic degradation of benzothiophene-2,3-dione. a) chromatograph and UV-Vis scan of authentic 2,2'-dithiosalicylic acid standard. b) diode array chromatograph at λ240 nm of solution. c) UV-vis scan of the other two disulfide products.

5.3.2 Affect of temperature, pH, and light on disulfide formation and decarbonylation

Figure 5.10 illustrates the proposed pathway for disulfide formation and subsequent decarbonylations. There are three known mechanisms for the decarbonylation of α-ketocarboxylic acids (March, 1985). The first mechanism is acid catalyzed, while the second mechanism is known to be light activated. These α-ketocarboxylic acids are also known to be thermally unstable and can spontaneously undergo a temperature dependant decarbonylation. The mechanism of this third decarbonylation is not totally understood. To differentiate among these three mechanisms of decarbonylation, and to characterize the oxidation of 2-mercaptophenylglyoxylate to the disulfide, the effect of pH, temperature and light were investigated.

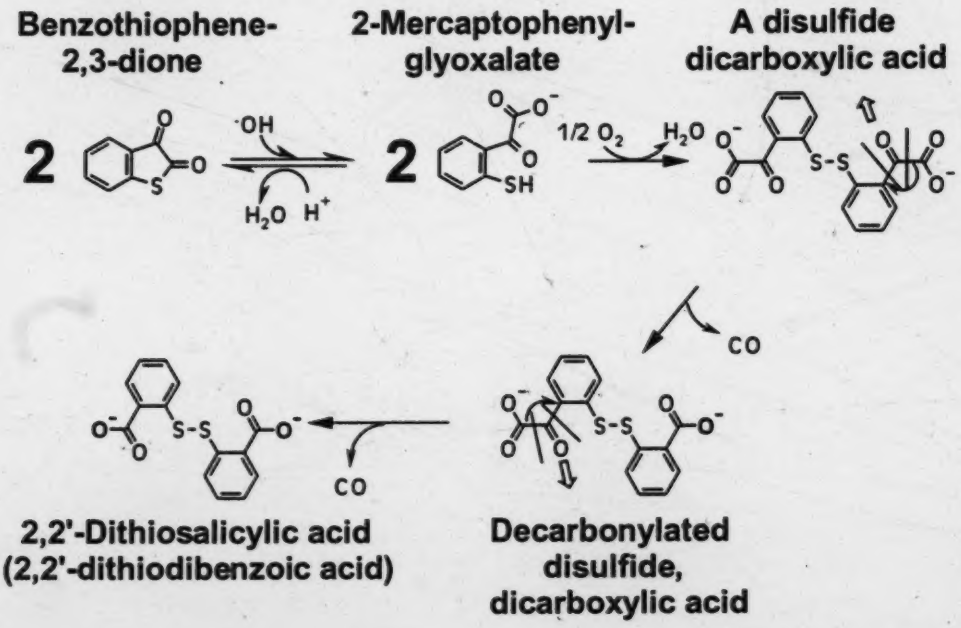

Figure 5.10 Formation of disulfides from 2-mercaptophenylglyoxalate and subsequent decarbonylation reactions.

As illustrated in Figure 5.11, benzothiophene-2,3-dione (peak A) was only stable to any extent at pH 1 incubation. At pH 3 and above, complete disappearance of the dione (peak A) was observed with formation of at least one of the disulfides (peaks C and D). At higher pH, the rate of decarbonylation appeared to increase as demonstrated by

increasing concentrations of the smaller disulfides at each pH increment. These findings are in contrast with an acid catalyzed mechanism of decarbonylation where it would have been expected that reducing the pH should have accelerated decarbonylations. It appeared that the decarbonylation reactions were quite complex in that at pH 3, compound E could be observed, but not at pH 5 or 7. At pH 11, the decarbonylation reactions appeared not to occur to any great extent as witnessed through a lack of smaller disulfides. It is apparent that at pH 11 that some other factor interferes with the decarbonylation reactions.

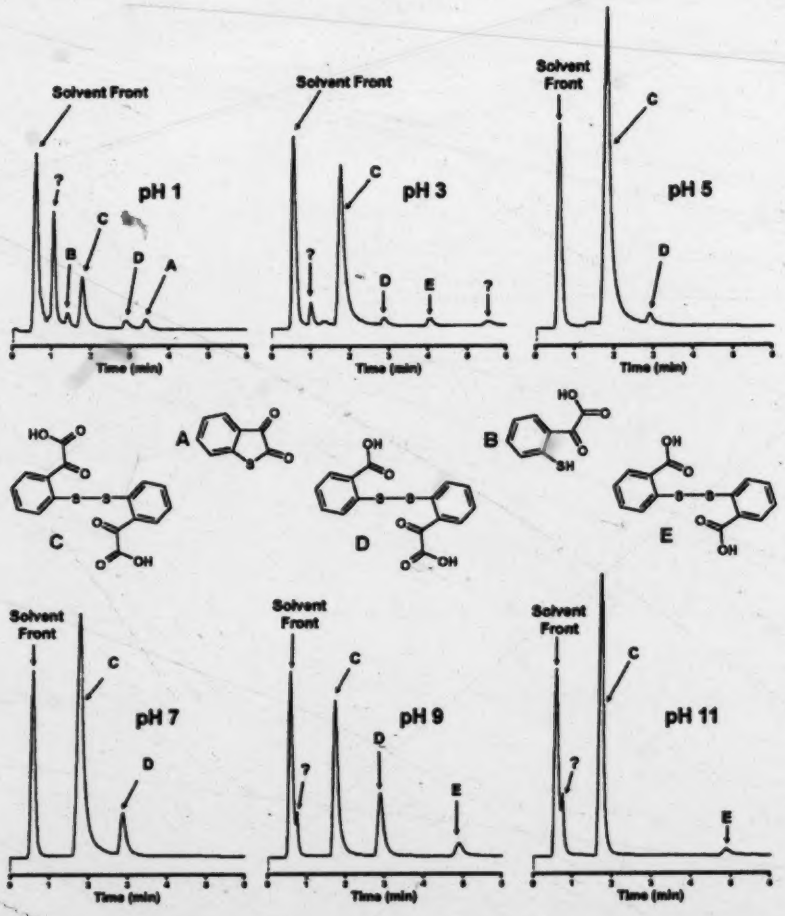

Figure 5.11 Effect of pH on disulfide formation as monitored through HPLC analysis at λ240nm of 1-month-old sterile incubations of the 2,3-dione in mineral salts medium.

In a subsequent experiment (Figure 5.12), both temperature and light exposure were tested for there effect on disulfide formation and decarbonylation. Over the course of the experiment, exposure to light appeared to increase the rate of loss of the first disulfide (peak C), but a corresponding increase in the second disulfide (loss of one carbonyl group, peak D) was not observed. Instead, an apparent new broad peak (?) was observed in all replicates, which remained unidentified. Decreasing the temperature of the experiment resulted in a delay in disulfide formation. It required 4 days at 4°C for oxidation of 2-mercaptophenylglyoxylate to the first disulfide (peak C) to complete (instead of 24-48h at 28°C). Subsequent decarbonylation reactions were also delayed by the decreased temperature. It appeared that temperature had the greatest affect on the rate of disulfide formation and decarbonylation.

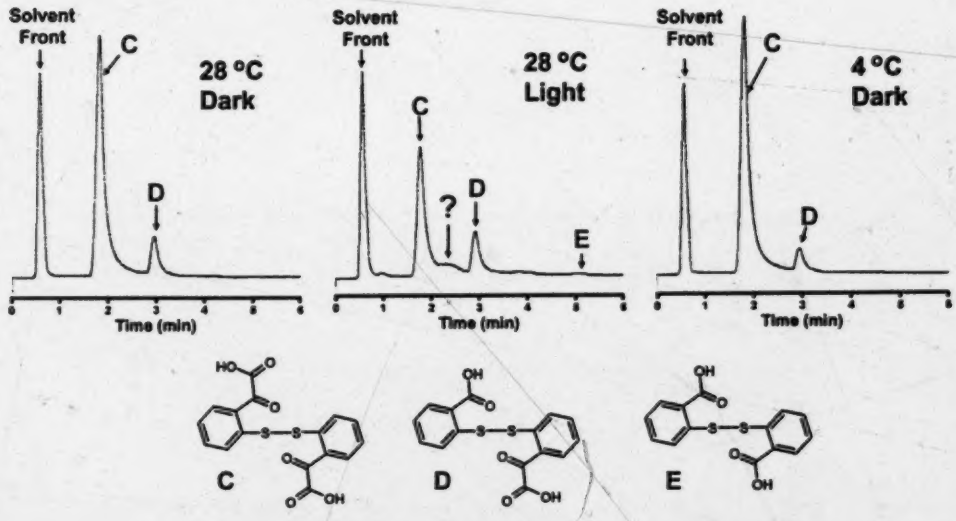

Figure 5.12 Effect of temperature and light exposure on disulfide formation as monitored through HPLC analysis at λ240nm of 18 day old sterile incubations of the 2,3-dione in mineral salts medium.

5.3.2 Bacterial disulfide production from dibenzothiophene

After the disulfides were observed as oxidation products of 2-mercaptophenylglyoxylate, it was hypothesized that the disulfides should be observed in bacterial cultures known to produce benzothiophene-2,3-dione during dibenzothiophene biodegradation. To test this hypothesis, *Pseudomonas* BT1d, known to use dibenzothiophene as sole carbon and energy source and produce small amounts of benzothiophene-2,3-dione as a metabolite, was grown on excess dibenzothiophene in mineral salts medium. After incubation, cultures were extracted under acidic (pH<1) conditions, subjected to diazomethane derivatization and analyzed by GC-MS. As shown in Figure 5.13, all three disulfides were observed in this extract. Although not quantitative, it is apparent that the disulfides constitute a significant proportion of the total metabolites formed.

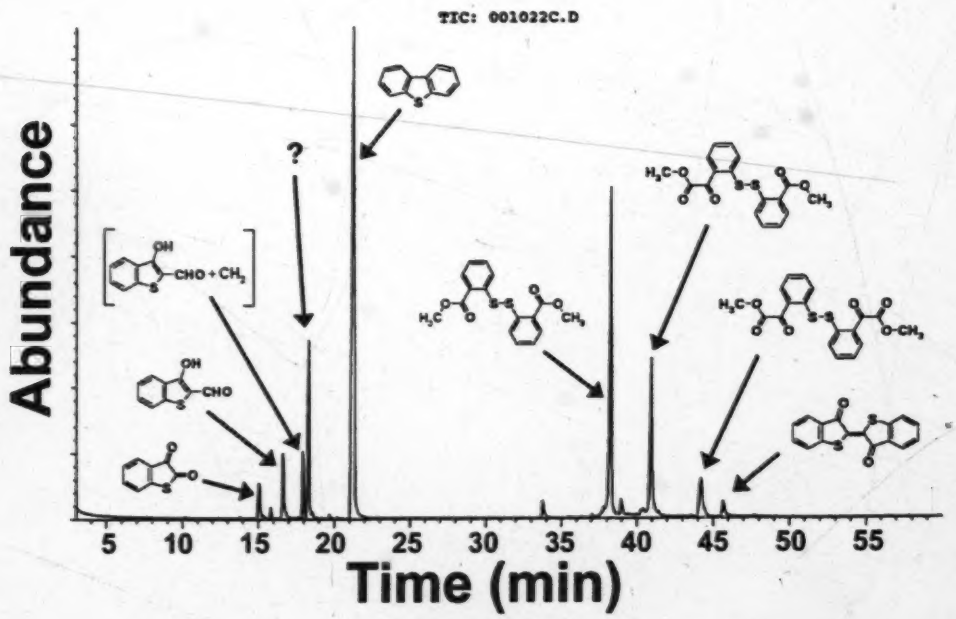

Figure 5.13 GC-MS total ion current chromatography of the diazomethane derivatized extract of a *Pseudomonas* BT1d culture grown on excess dibenzothiophene in mineral salts medium. (The CH_2 addition to HFBT is due to the diazomethane reaction.)

Subsequently, three other *Pseudomonas* strains (BT1, W1, and F) known to co-metabolize dibenzothiophene while growing on 1-methylnaphthalene, along with BT1d were used in dibenzothiophene biodegradation experiments aimed at quantifying the amount of disulfides produced during dibenzothiophene biodegradation. After 14 days, the cultures were acidified and extracted. Unfortunately, the large amounts of biomass produced from the three cultures supplemented on 1-methylnaphthalene resulted in the formation of a large interphase during solvent extraction. Precipitating disulfides were observed to accumulate in the interphase and could not be recovered for derivatization. Thus the identification and quantification of the disulfides from these other three strains failed. Even in the case of strain BT1d, where no 1-methylnaphthalene was used, significant disulfide was presumed lost in the interphase present.

5.3.2 Biodegradation of 2,2'-dithiosalicylic acid

This investigation clearly revealed that benzothiophene-2,3-dione and 2-mercaptophenylglyoxylate would not be expected to exist for any length of time at neutral pH in anaerobic environments. It was decided to subject 2,2'-dithiosalicylic acid, the last recognized oxidation and decarbonylation product (Compound E Figure 5.10), to biodegradation studies. This compound was commercially available while the other two disulfides were not.

Two garden soils were inoculated into mineral salts medium containing 2,2'-dithiosalicylic acid (100 mg/L). After enrichment, stable biodegrading mixed cultures were obtained. As shown in Figure 5.14a, both cultures showed removal of the substrate over a 1 month incubation. Figure 5.14b demonstrates that a drop in pH was observed in both cultures. This decrease in pH can be explained by equation 5.1.

$$2\ C_{14}H_{10}S_2O_4 + 35\ O_2 \rightarrow 28\ CO_2 + 6\ H_2O + 4\ H_2SO_4 \quad \text{[equation 5.1]}$$

It can be noted that biodegradation of the 2,2'-dithiosalicylic acid results in the production of 2 mol of sulfuric acid for every 1 mol of substrate mineralized. Subsequent analysis of the cultures for sulfate release revealed that Garden Culture 1 released 83% of the substrate sulfur as sulfate while Garden Culture 2 released 84%.

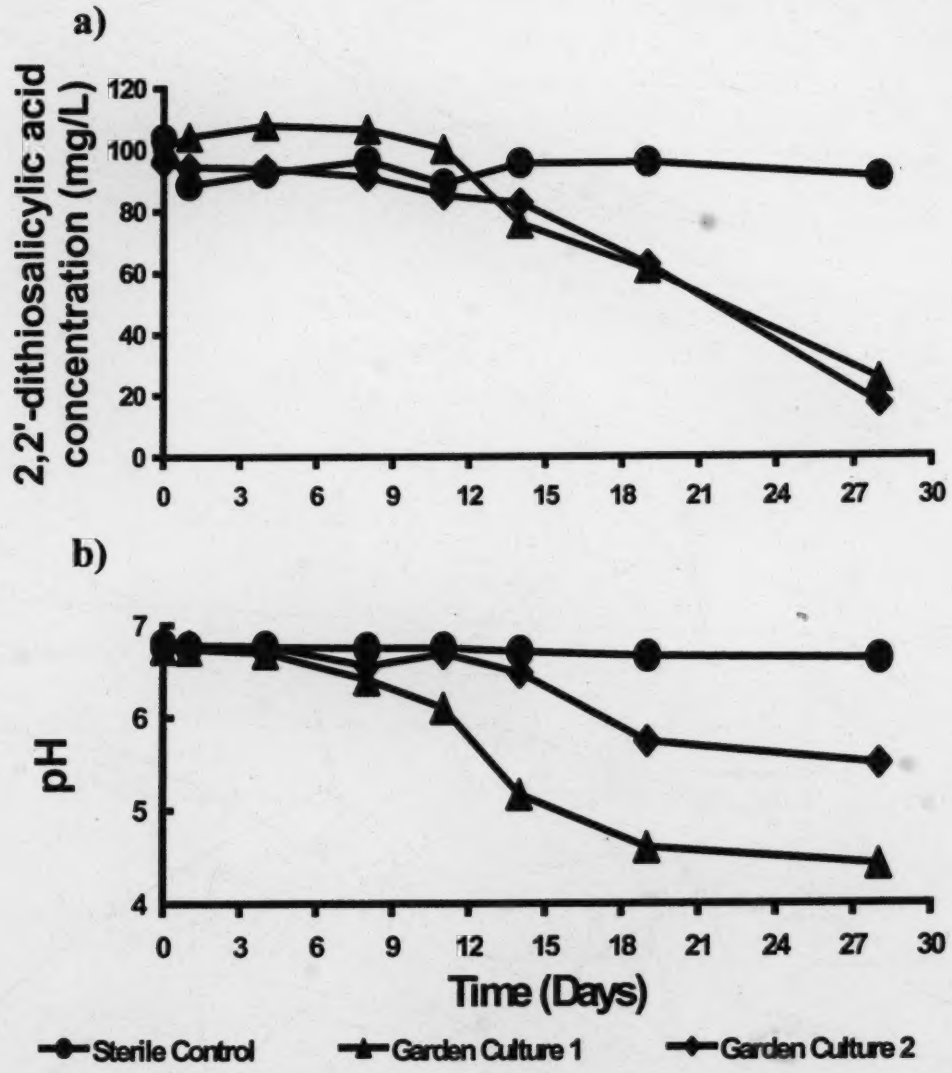

Figure 5.14 Biodegradation of 2,2'-dithiosalicylic acid in mineral salts medium by two mixed bacterial communities (a) and pH change in the cultures (b)

To understand the extent of 2,2'-dithiosalicylic acid biodegradation by the two garden cultures, a carbon balance was attempted. Table 5.1 shows that triplicate cultures of both mixed populations were able to demonstrate biotransformation or mineralization of 2,2'-dithiosalicylic acid. In three of the cultures, Garden 1-1, 2-2, and 2-3, most of the starting substrate was biotransformed. Other cultures such as Garden 1-2, demonstrated

some substrate removal (Figure 5.15, Table 5.1), but biodegradation was interrupted for some unknown reason. The other three cultures show significant mineralization. The variation among cultures demonstrates that the mixed cultures may not have reached a stabilized enrichment level and that not all factors affecting the growth of these cultures have been determined.

Table 5.1 Carbon balance for two 6-week-old garden soil cultures grown on 2,2'-dithiosalicylic acid and incubated in triplicate.
(*Origional substrate carbon content = 305 µmol)

Culture	Substrate carbon remaining (µmol)	Carbon dioxide (µmol)	Percent carbon released as CO_2	Unidentified dissolved organic carbon (µmol)	Total (µmol)	Percent recovery
Garden 1-1	0	60	20	250	310	100
Garden 1-2	200	89	30	43	340	110
Garden 1-3	270	0	0	0	270	88
Garden 2-1	0	210	70	97	310	100
Garden 2-2	0	1	0	280	280	91
Garden 2-3	0	0	0	280	280	90

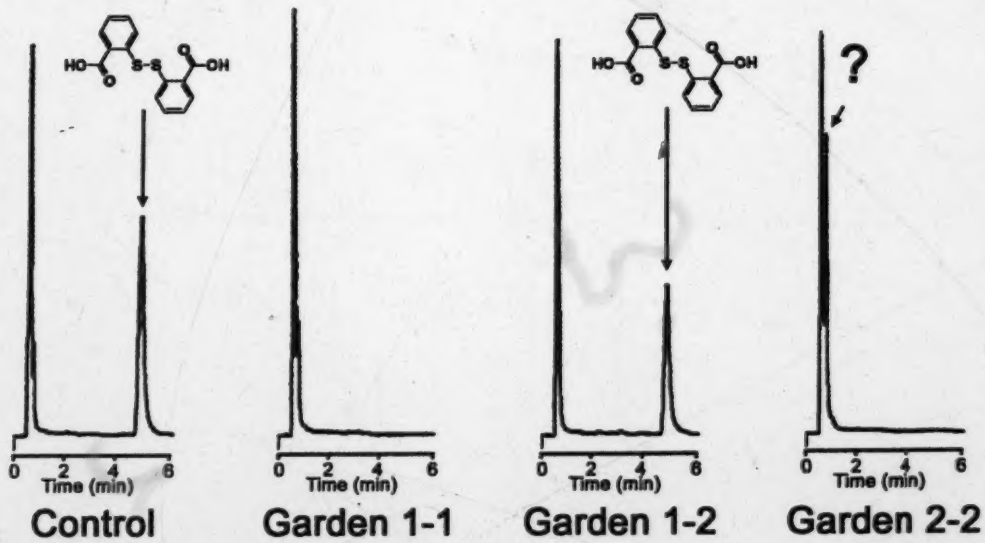

Figure 5.15 2,2'-Dithiosalicylic acid degrading mixed cultures enriched from garden soil after 6 weeks of incubation in mineral salts medium.

Four distinct colony types which had been isolated on PCA plates from the mixed garden soil cultures capable of biodegradation were reintroduced into 2,2'-dithiosalicylic acid containing mineral salts medium. Of the four distinct colony types, only two retained the ability to remove the 2,2'-dithiosalicylic acid from the medium. When these degrading cultures were again streaked on PCA agar, it was found that the cultures were not pure and each consisted of at least two major colony types. Further efforts to separate the colonies and establish isolated bacterial strains capable of removing 2,2'-dithiosalicylic acid were unsuccessful. It was possible that a combination of more than one bacterial population was necessary to maintain biodegradation capability.

5.4 DISCUSSION

This investigation has clearly indicated that 2-mercaptophenylglyoxylate may be a major product of dibenzothiophene biodegradation through the Kodama pathway. This is apparent because of the detection of significant amounts of the three disulfides in extracts of *Pseudomonas* BT1d grown on dibenzothiophene (Figure 5.13). This observation of the oxidation of 2-mercaptophenylglyoxalate to form the large disulfide and the subsequent decarbonylations, is the first report of the larger two disulfides formed in dibenzothiophene degrading cultures.

An earlier report by Finkel'stein *et al.* (1997) reported the presence of 2,2'-dithiosalicylic acid as a product of dibenzothiophene bacterial biodegradation, but their report implied that the disulfide was formed through the oxidation of 2-mercaptobenzoic acid. During the course of this investigation, it was possible that a small amount of the 2-mercaptophenylglyoxalate was decarbonylated before oxidation to form some of the disulfides. However, HPLC analysis clearly revealed that the vast majority of the 2-mercaptophenylglyoxalate was rapidly oxidized to form the disulfide before decarbonylation could occur.

Previous studies such as those conducted by Kropp *et al.* (1996) and Bressler and Fedorak (2001) failed to recognize the production of these disulfides. This lack of recognition can be easily explained by differences in sample preparation. Earlier studies of dibenzothiophene biodegradation relied on organic extraction conditions including acidification to a pH around 2. Although this pH would generally protonate and extract

most carboxylic acids, to protonate both carboxylic groups on the disulfides, a much lower pH is required. One other technical consideration in the identification of the disulfides, as was necessary for the identification of thioindigo by Bressler and Fedorak (2001), was the elevated GC temperature programs. Even with column temperatures of 280°C, the methyl esters of the disulfides had GC retention times greater than 40 min, and without derivatization they would not pass through the GC at all. It was the use of HPLC, and especially HPLC-MS, which provided the initial indications that the disulfides existed.

Another problem with routine sample preparation in past experiments has been the use of inorganic drying agents such as anhydrous sodium sulfate. During the analysis of the disulfides formed during this investigation, it was apparent that upon acidification the disulfide became insoluble in the aqueous phase and only sparingly soluble in all organic phases commonly used for extractions. The disulfides were observed to precipitate out of solution, often associating with the interphase or small emulsion layers. If a bacterial culture was used to generate the disulfides (Figure 5.13) then a substantial amount of the disulfides associated with the biomass at the interphase during extraction. Quantitative separation of the disulfides from the interphase for diazomethane derivatization was impossible, preventing accurate determination of the amount of dibenzothiophene converted to disulfides.

If the organic phase was dried with an inorganic drying agent, the disulfides were lost to associations with the drying agents (Figure 5.6b). The disulfides could not be quantitatively derivatized due to the excess aqueous phase remnants that were collected along with the interphase material when drying agents were not used. Previous investigations, such as Kropp et al. (1996), routinely used drying agents to prepare organic extracts for further analysis and thus would never have seen significant quantities of the disulfides.

2-Mercaptophenylglyoxalate biodegradation has not been previously reported. There have been numerous reports describing the detection of benzothiophene-2,3-dione and its methyl analogs in acidified culture extracts. Reports by Bohonos *et al.* (1977), Eaton and Nitterauer (1994), and Fedorak and Grbić-Galić (1991) revealed that the 2,3-dione had been found in cultures degrading benzothiophene. The same study by Bohonos

et al. (1997), and that by Kropp *et al.* (1997a) revealed that the 2,3-dione was also found in dibenzothiophene degrading cultures. The common appearance of these compounds indicates that the 2-mercaptophenylglyoxalates must be important intermediates in the biodegradation of benzothiophenes and dibenzothiophenes.

Reports have also shown that methylbenzothiophene-2,3-diones are observed in cultures degrading methylbenzothiophenes (Kropp *et al.* 1994a; Saftić *et al.* 1992) and in dimethylbenzothiophenes (Kropp *et al.* 1996). Methyldibenzothiophenes (Saftić *et al.* 1993) and dimethyldibenzothiophenes such as 2,8-dimethyldibenzothiophene (Kropp *et al.* 1997b) have been shown to produce methylbenzothiophene-2,3-diones while 3,4-dimethyldibenzothiophene (Kropp *et al.* 1997b) produced dimethyldibenzothiophene-2,3-diones. Recent unpublished findings by Leon Lau in our research group confirmed that 7-methylbenzothiophene sulfone underwent the analogous ring opening equilibrium to the 3-methyl-2-mercaptophenylglyoxylate which subsequently oxidized to give the corresponding disulfide. Subsequent decarbonylations resulting in 3,3'-dimethyl-2,2'-dithiosalicylic acid were also observed. Based on the earlier reports (Kropp *et al.* 1994a, 1996, 1997b; Saftić *et al.* 1992, 1993), it would be expected that a range of substituted 2-mercaptophenylglyoxylates, and substituted salicylic acids would be found in these types of environments due to the range of substituted dibenzothiophenes found in petroleum known to undergo biodegradation. Substituted 2-mercaptophenylglyoxylates and substituted salicylic acids would be expected to oxidize to give an assortment of disulfide products, limited only by the number of substituted dibenzothiophenes found in the original petroleum mixture. These findings, in conjunction with the findings of this investigation, would indicate that a mixture of disulfide compounds would be found, due to abiotic side reactions, in petroleum contaminated environments where biodegradation processes were occurring.

The experiments involving the effect of pH on disulfide formation demonstrate that at pH 3 and above, the disulfide formation is accelerated as the pH is increased. This phenomenon is easily explained as a result of the 2,3-dione–mercaptophenylglyoxylate ring closure equilibrium. At very low pH, the equilibrium shifts to where the majority of the mass exists as the closed ring form. Benzothiophene-2,3-dione does not play a role in the disulfide formation. At pH 3 and above, most of the molecules exist as the open ring

form (2-mercaptophenylglyoxylate) and thus disulfide formation can occur. The only other factor observed to affect disulfide formation is temperature. During experiments testing the effect of temperature on decarbonylation and disulfide formation, it was observed that an incubation temperature of 4°C drastically slowed the formation of the disulfide. This slowing of the oxidation reaction is expected and would not be anticipated to change the final outcome of the oxidation reactions.

One of the more interesting findings of this study was the decarbonylation reactions themselves. Upon reviewing the literature, there are three main mechanisms which could be used to explain the observed decarbonylation mechanisms. The first mechanism would be a light activated decarbonylation. Although this type of reaction would seem likely, the light exposure experiment clearly showed this mechanism not to be the major contributor. The second possible mechanism of decarbonylation would be an acid catalyzed decarbonylation. Again this mechanism can be eliminated for the most part because the pH experiments illustrated that the rate of decarbonylation increases at higher pH, and at very low pH the 2,3-dione predominance would not allow the disulfide formation to occur to any great extent. Instead the most likely mechanism of decarbonylation is that referred to by March (1985). March notes that α-keto acids and α-keto esters can be decarbonylated by simple heating, but that the mechanisms are not known (Figure 5.10). By eliminating the first two mechanisms of decarbonylation through experimentation, it must be assumed that the decarbonylation of the disulfides occurs through this third unknown mechanism.

This investigation has shown that benzothiophene-2,3-dione is extremely unstable when in solution under all but the most reduced and acidic conditions. It would not be expected that the 2,3-dione would accumulate in the environment due to its extreme instability and therefore one would not expect to isolate bacterial strains specializing in its biodegradation. Instead, investigations into understanding the fate of the disulfides will lead to discovering more products of dibenzothiophene biodegradation.

This study has demonstrated that benzothiophene-2,3-dione only exists at low pH. It is possible that 2-mercaptophenylglyoxylate is the actual metabolite of dibenzothiophene biodegradation and the 2,3-dione is formed only under the acidic conditions used for sample extraction. A second possibility supported by evidence in

Chapter 4 is that HFBT, an earlier metabolite in dibenzothiophene biodegradation condenses to yield thioindigo which subsequently is oxidized to yield benzothiophene-2,3-dione. This type of oxidative cleavage of thioindigo to yield benzothiophene-2,3-dione has been reported under ozonolysis (Rajopadhye and Popp 1988). Once the 2,3-dione was formed, it would then spontaneously open to yield 2-mercaptophenylglyoxylate.

2-Mercaptophenylglyoxylate is not likely to accumulate as a biodegradation product because it oxidizes to a disulfide. It could safely be assumed that in the environment, any biodegradation resulting in 2-mercaptophenylglyoxylate formation would inevitably result in the formation of all three disulfides, except under the most reduced or acidic environments.

If the disulfides are indeed major metabolites of dibenzothiophene biodegradation, it is important to determine whether the disulfides are biodegradable. This report is believed to be the first to assess the biodegradation of the disulfides formed through 2-mercaptophenylglyoxalate oxidation. Microbial populations were enriched which had the ability to mineralize 2,2'-dithiosalicylic acid, to the extent that sulfate was released and significant carbon dioxide release was monitored, were isolated from two different garden soil inoculations. The release of significant amounts of sulfur as sulfate, 83% and 84% by the two garden cultures, indicates a significant amount of mineralization. It is assumed that the thiophene ring would have to have been opened. Any product left after the sulfur was removed would be expected to be oxidized and perhaps highly biodegradable. These findings indicate that the ability to degrade the disulfides does not appear to be that scarce. Unfortunately, one complication with biodegradation of 2,2'-dithiosalicylic acid is that significant amounts of acid production occur causing a decrease in medium pH. If unchecked this drop in pH often results in death of the cultures. Future investigations into the biodegradation of these disulfides will have to ensure that cultures will have strong buffers to prevent drastic pH drops during biodegradation.

The autoxidation of aromatic thiols to disulfides as demonstrated by salicylic acid which is known to autoxidize to 2,2'-dithiosalicylic acid at neutral pH have been shown to generate active oxygen species such as O_2^- and H_2O_2 (Munday 1985a; 1985b). This

generation of active oxygen species by 2-mercaptophenylglyoxylate was not investigated here but would be predicted based on other known thiol autoxidation reactions (Munday 1985a, 1985b). If active oxygen is produced during the formation of the disulfides, then the toxicity created could explain why efforts to isolate a bacterial consortium able to degrade a mixture of disulfides, formed by including benzothiophene-2,3-dione as a substrate, failed. This principal would also suggest that biodegradation of dibenzothiophene and other components of petroleum contaminants resulting in thiol metabolites capable of autoxidation to disulfides, would generate increased toxicity in the environment.

The fact that the disulfides can be identified in *Pseudomonas* BT1d cultures clearly indicates the disulfides are indeed novel products of the biodegradation of dibenzothiophene. Figure 5.16 illustrates an updated dibenzothiophene biodegradation pathway accounting for the formation of the disulfides from 2-mercaptophenylglyoxylate. With this information, it should be possible to assemble a microbial consortium that could mineralize dibenzothiophene and some substituted dibenzothiophenes to HFBT and finally to 2-mercaptophenylglyoxylate, or analogous substituted metabolites. Subsequent abiotic reactions of 2-mercaptophenylglyoxylate or analogs would then be expected to oxidize to the disulfides. After subsequent abiotic decarbonylations, the microbial consortium could be expected to mineralize the 2,2'-dithiosalicylic acid and analogs to carbon dioxide and sulfate. This suggested pathway is summarized in Figure 5.16.

One of the major findings of this study is that major metabolites of dibenzothiophene biodegradation can be identified. Even with the copious amounts of information on dibenzothiophene biodegradation, most researchers have ignored the search for larger metabolites with the exception of Kropp et al. (1994b) and Bressler and Fedorak (2001). Further investigations are required to determine the actual fate of the disulfides upon subsequent biodegradation. Findings accumulated during the attempted dibenzothiophene biodegradation sulfur mass balance experiments demonstrated that sulfur contained within the disulfides, as well as sulfur within thioindigo, clearly represent a significant proportion of the initial dibenzothiophene sulfur. Further research and development of analytical techniques are needed to achieve an accurate understanding of the proportion of higher molecular weight metabolites formed. These

investigations clearly indicate that the accumulation of these high molecular weight products is much more significant than first realized.

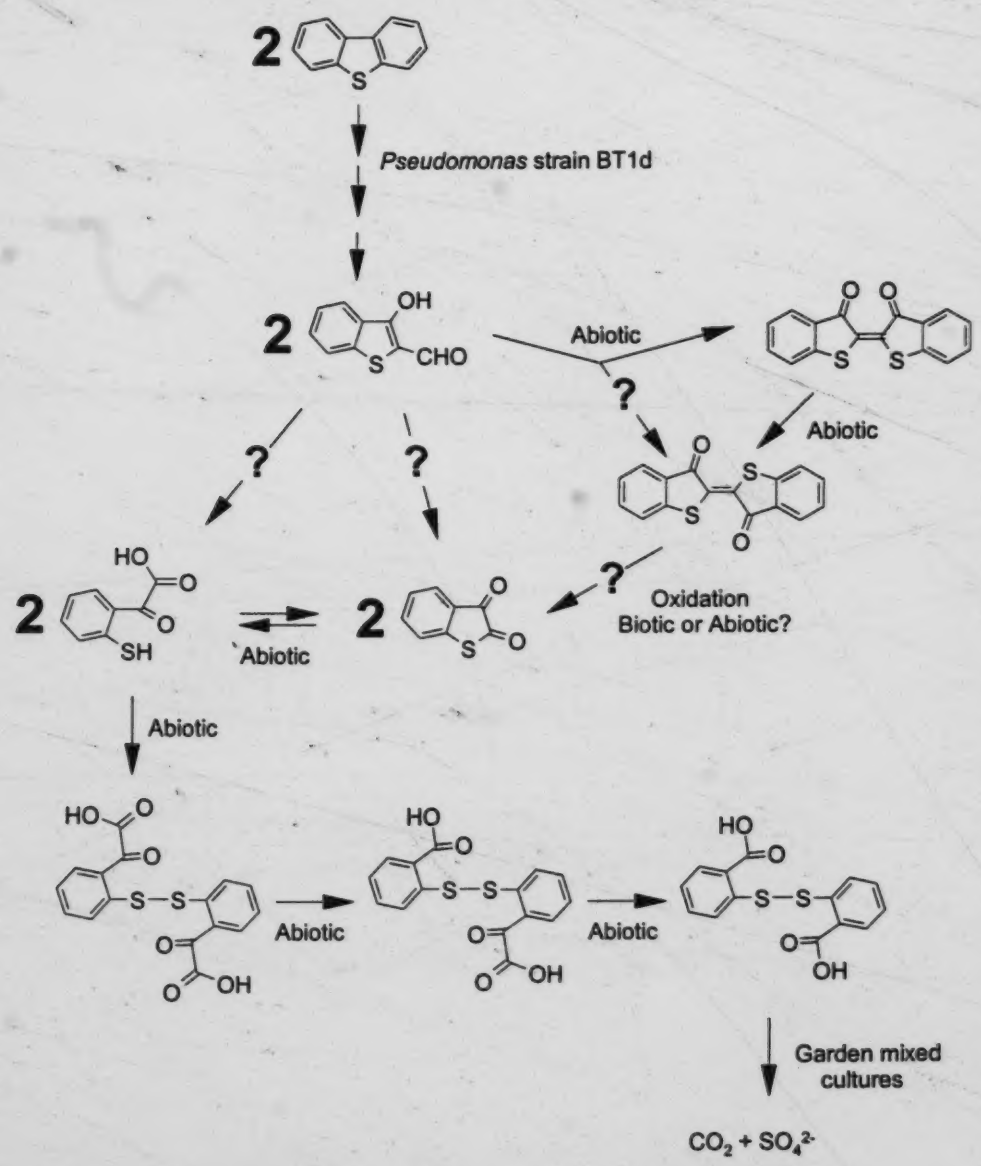

Figure 5.16 Summary of the possible abiotic and biotic reactions leading to the formation of 2,2'-dithiosalicylic acid from dibenzothiophene

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6. OVERALL DISCUSSION AND SUGGESTIONS FOR FURTHER RESEARCH*

6.1 OVERALL DISSCUSSION

This dissertation has not discussed all aspects of the biodegradation of sulfur heterocycles. Indeed, there are scores of publications on biodesulfurization and the microbial metabolism of dibenzothiophene and benzothiophene which have not been cited, but none of these provide additional insight into biodegradation pathways, how ring cleavage occurs or what the final fate of the known biodegradation metabolites is. There is still a large void in our knowledge of the mechanisms involved in these biodegradation pathways. Among the many examples of ring cleavage, few have been studied in detail.

There are two major objectives driving the studies of the microbial metabolism of organosulfur compounds. One is the quest to develop a process for biodesulfurization of fossil fuels. The other is to understand the fates of organosulfur compounds in petroleum- or creosote-contaminated environments, which is important in the assessment of bioremediation activities. Of course, the goal of the biodesulfurization process is to leave the carbon skeletons of the organosulfur compounds intact, whereas the aim of any bioremediation process is to mineralize all of the contaminant compounds, including the organosulfur compounds. This dissertation has focused on the biodegradation of metabolites that would be expected to form under conditions of environmental biodegradation and not desulfurization pathways.

There is irrefutable evidence that aerobic microbial metabolism can result in the release of the sulfur atom from dibenzothiophene and related compounds. This has been demonstrated by several research groups (Isbister *et al.* 1988; Kayser *et al.* 1993; van Afferden *et al.* 1993; Lee *et al.* 1995; Gray *et al.* 1996; Ohshiro *et al.* 1996), however, the evidence for the release of sulfur from condensed thiophenes under anaerobic conditions is much weaker. Only a few groups have reported this phenomenon (Kurita *et al.* 1971;

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* Portions of this chapter have been previously published.
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Kim *et al.* 1990a, 1990b; Lizama 1995), and this slow process appears to be difficult to demonstrate. Table 6.1 summarizes the aerobic microbial metabolism of many types of organosulfur compounds. Some common themes are evident from the data in Table 6.1. For example, in all cases, oxidation of the organosulfur compound occurs before ring cleavage.

The most conclusive evidence demonstrating thiophene ring cleavage of benzothiophene is from the work of Eaton and Nitterauer (1994) who showed the formation of 2-mercaptophenylglyoxalate, which cyclized to benzothiophene-2,3-dione upon acidification of the culture medium. Oxidation of the thiophene ring by a dioxygenase has been reported to form *cis*-2,3-dihydroxy-2,3-dihydrobenzothiophene, which is transformed spontaneously and/or enzymatically to 2-mercaptophenylglyoxalate (Eaton and Nitterauer 1994). The net result of the oxidation of C2 and C3 in benzothiophene is the weakening of the bond between C2 and the sulfur atom, which leads to ring cleavage. Others have observed 2,3-diones from methyl- (Saftić *et al.* 1992; Kropp *et al.* 1994) and dimethylbenzothiophenes (Kropp *et al.* 1996). Thus, this mode of oxidation leading to thiophene ring cleavage appears to be common.

2,3-Diones cannot be formed in this manner if there is a methyl group on C2 and/or C3 (Fedorak and Grbić-Galić 1991; Saftić *et al.* 1992). In studies with 3-methylbenzothiophene, Selifonov *et al.* (1996) demonstrated that naphthalene 1,2-dioxygenase behaves as a sulfoxygenase oxidizing this compound to 3-methylbenzothiophene sulfoxide. Fedorak and Grbić-Galić (1991) provided evidence that this sulfoxide was an intermediate in the formation of 3-methylbenzothiophene sulfone, much the same as dibenzothiophene sulfoxide is an intermediate in the formation of dibenzothiophene sulfone by the 4S pathway (Figure 1.8). Chapter 2 of this thesis reports the isolation of a bacterial culture that grows on 3-methylbenzothiophene sulfone and releases sulfate from this compound. Hence, the sulfone is susceptible to ring cleavage, but the exact mechanism for this is not completely known. This dissertation has added some new insight into the mechanisms of this ring cleavage, however. In Chapter 3, 2,3-dihydro-2-hydroxybenzothiophene sulfone is identified as a product of benzothiophene sulfone biodegradation. This new metabolite may represent another intermediate in the thiophene ring cleavage pathway, or it could be a dead-end metabolite.

Table 6.1 Summary of aerobic studies of microbial metabolism of organosulfur compounds.

Organosulfur compounds	Microbial transformation process and/or metabolites detected	Cleavage of sulfur-containing ring demonstrated
Alkylthiolanes	- Side chain degraded to thiolane carboxylic acid (1)[a]	No
Alkylthiophenes	- Side chain degraded to thiophene carboxylic acids (2, 3)	Yes, from some thiophene carboxylic acids (3, 4, 5)
Sulfolane	- Process unknown, but sulfate detected (6)	Yes (6)
Benzothiophenes	- Oxidized to 2,3-diones which exist as mercaptophenylglyoxalates at neutral pH (7, 8, 9, 10)	Yes (7). No, but the 2,3-diones detected (8, 9, 10)
	- Oxidized to sulfones (9,10, 11)	Yes, from sulfone (12)
Dibenzothiophenes	- Oxidation of the sulfur atom leading to desulfurization by 4S pathway producing substituted biphenyl (13, 14, 15, 16, 17)	Yes (13, 14, 15, 16, 17)
	- Degradation of homocyclic rings to give 2,3-diones which exist as mercaptophenylglyoxalates at neutral pH (18, 19, 20)	No, but the 2,3-diones detected (18, 19, 20)
	- Oxidation of the sulfur atom, followed by oxidation of the homocyclic ring (21, 22)	Yes (21, 22)
Benzonaphtho-thiophenes	- Oxidation of the sulfur atom leading to desulfurized by 4S pathway leaving α-hydroxy-β-phenylnaphthalene (17)	Yes (17)

[a]Reference number

1 Fedorak et al. (1988) 2 Fedorak & Beakman (1992) 3 Fedorak et al. (1996)
4 Cripps (1973) 5 Kanagawa & Kelly (1987) 6 Chou & Swatloski (1983)
7 Eaton & Nitterauer (1994) 8 Bohonos et al. (1977) 9 Saftić et al. (1992)
10 Kropp et al. (1994a) 11 Selifonov et al. (1996) 12 Bressler & Fedorak (1999)
13 Isbister et al. (1988) 14 Kayser et al. (1993) 15 Lee et al. (1995)
16 Gray et al. (1996) 17 Ohshiro et al. (1996) 18 Saftić et al. (1993)
19 Kropp et al. (1996) 20 Kropp et al. (1997) 21 van Afferden et al. (1990)
22 van Afferden et al. (1993)

This metabolite may indicate that oxidation to the 2,3-dione is not totally independent from the oxidation of the sulfur atom to the sulfone group. Unfortunately, oxidation of 3-methylbenzothiophene sulfone to 2,3-dihydro-2-hydroxy-3-methylbenzothiophene sulfone was not observed. However, further investigations are required to determine the role of this oxidation to a dihydrohydroxybenzothiophene in the thiophene ring biodegradation pathway.

Studies with dibenzothiophene and related compounds show that the initial oxidation of the sulfur atom via the modified 4S pathway leads to cleavage of the thiophene ring prior to the selective removal of the sulfur atom (Table 6.1). The only reported biodegradation of a benzonaphthothiophene occurs via this pathway (Ohshiro *et al.* 1996), but no investigations into the biodegradation of benzonaphthothiophene sulfones have been reported. van Afferden *et al.* (1993) demonstrated that some bacteria initially oxidize the sulfur atom in dibenzothiophene and then they oxidize the homocyclic ring through a 4,4a angular attack pathway, which leads to cleavage of the thiophene ring.

It appears that the thiophene ring of dibenzothiophene can also be broken by a mechanism analogous to that of benzothiophene via the same mechanism that yields 2-mercaptophenylglyoxalates, because 2,3-diones have been found in extracts of acidified culture supernatants (Table 6.1). The 2-mercaptophenylglyoxalates likely arise from the metabolism of 3-hydroxy-2-formylbenzothiophene, an observation strengthened by Chapters 4 and 5 of this thesis which demonstrate the production of the 2,3-dione from 3-hydroxy-2-formylbenzothiophene, but the mechanism of transformation remains unknown.

Although the modified 4S pathway is well-understood, there is clearly much more research needed to elucidate how ring cleavage in sulfur heterocycles happens. The lacking information is of special interest to those concerned with the biodegradation of sulfur heterocycles in contaminated environments. These investigations are hampered by the lack of commercially available compounds of interest. For example, although a few methylbenzothiophenes are commercially available, none of the methylated dibenzothiophenes or the known metabolites such as thiolane-2-carboxylic acid, 3-hydroxy-2-formylbenzothiophene and benzothiophene-2,3-dione are available from

chemical companies. Indeed, these metabolic studies would be much easier if radiolabeled sulfur heterocycles were readily available. Also, the cleavage of the sulfur heterocycles leads to very polar organosulfur compounds, which are not amenable to extraction into organic solvents for analysis by gas chromatography-mass spectrometry (GC-MS). In spite of these obstacles, the literature reviewed for this thesis indicates that our understanding of ring cleavage in sulfur heterocycles is slowly increasing.

One reason that some of these chemicals are not commercially available may be that often times they are highly chemically unstable. Chapters 4 and 5 of this investigation dealt with the study of benzothiophene-2,3-dione and 3-hydroxy-2-formylbenzothiophene. Both of these compounds were found to be chemically unstable under certain conditions making purification and biodegradation study quite difficult. In both cases, extreme amounts of time and effort were required to characterize the chemical properties of these compounds so that purified substrate could be generated and utilized properly for biodegradation studies.

This dissertation has demonstrated that many of the known condensed thiophene metabolites, which have been previously identified, would not be expected to be recalcitrant in the environment. Chapters 4 and 5 both clearly demonstrated that benzothiophene-2,3-dione and 3-hydroxy-2-formylbenzothiophene (HFBT) are both subject to abiotic oxidations and condensations. This dissertation demonstrated that every one of the recognized condensed thiophenes that was tested for biodegradation was susceptible to microbial attack. This was accomplished through either identifying metabolites (Chapter 2) or by monitoring mineralization in terms of sulfate and CO_2 release.

One reoccurring theme in biodegradation studies of the oxidized metabolites of dibenzothiophene and benzothiophene, that was previously often overlooked, is that abiotic oxidation and condensation products significantly larger than the starting substrates, can be generated (Figure 6.1). Two examples of Diels-Alder condensations have been reported including benzonaphthothiophene from benzothiophene sulfoxide (by Kropp *et al.* 1994b) and benzonapthothiophene sulfone from benzothiophene sulfone (Chapter 3 of this dissertation). Chapter 4 of this dissertation also reports the condensation of 3-hydroxy-2-formylbenzothiophene to give thioindigo as an abiotic

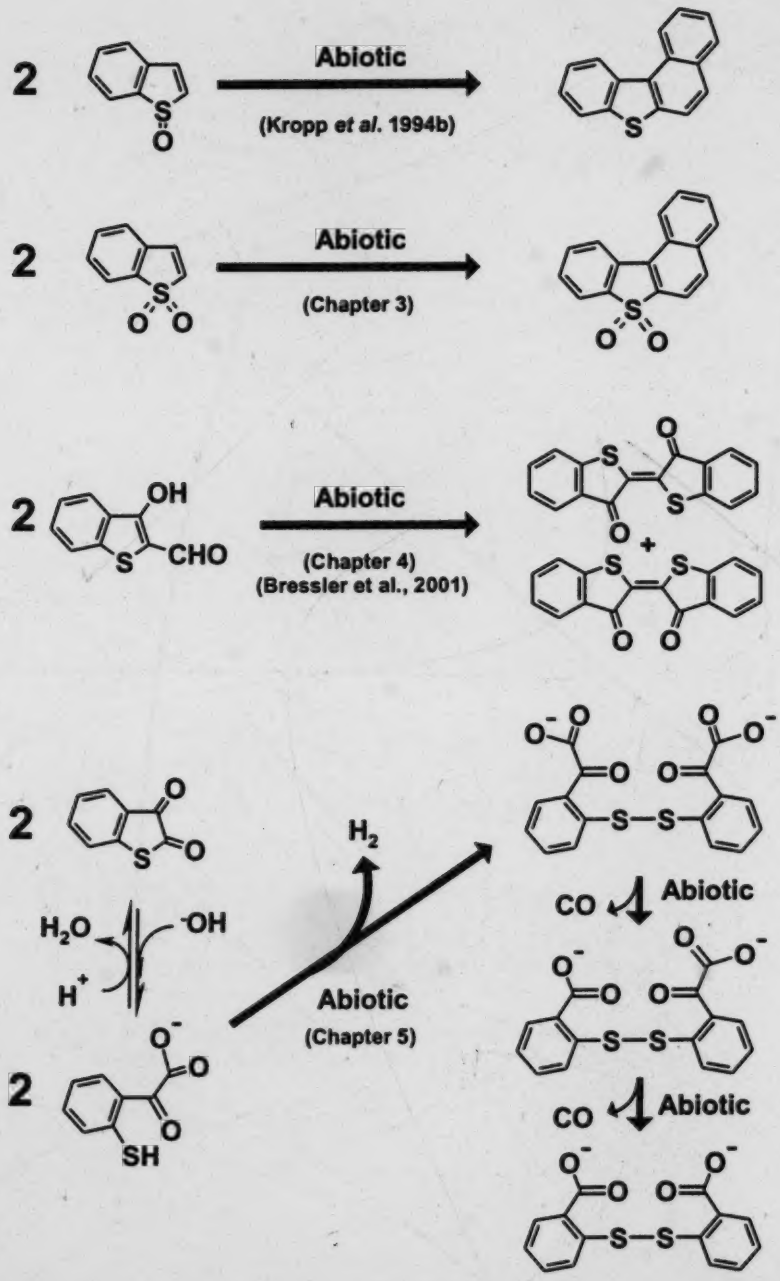

Figure 6.1 Oxidation and condensation reactions of condensed thiophene metabolites.

condensation product. Abiotic oxidation of 2-mercaptophenylglyoxylate to give a large disulfide was reported in Chapter 5 along with the description of the subsequent abiotic decarbonylations resulting in the production of 2,2'-dithiosalicylic acid. Most of the body of literature on the biodegradation of condensed thiophenes, sulfur heterocycles in general, and polycyclic aromatic hydrocarbons focused on the search for, and identification of, smaller more oxidized metabolites. Some of these compounds, such as benzothiophene-2,3-dione (or 2–mercaptophenylglyoxylate) are only found in trace quantities due to their unstable chemical properties. Methodologies and experimental approaches used by previous investigations would be expected to fail in the search for these larger oxidation and condensation products. It is only after experimental protocols are adjusted to search for these larger products that they can be found. Examples of this are increased temperature programs for gas chromatography (GC) protocols, and more stringent extraction conditions to search for highly acidic compounds.

This thesis has clearly demonstrated that oxidation and condensation reactions resulting in larger products can clearly play a major role in determining the fate of biodegraded sulfur heterocycles. It would not be surprising if similar reactions were found to be common for other chemical classes such as polycyclic aromatic hydrocarbons, nitrogen heterocycles, and oxygen heterocycles.

6.2 SUGGESTIONS FOR FURTHER RESEARCH

This investigation has clearly demonstrated that the oxidized biodegradation metabolites of benzothiophene and dibenzothiophene can be subject to further biodegradation. One overlying complication however is that as these condensed thiophenes are biodegraded, two main trends are observed. In the first trend, the metabolites tend to get smaller and more polar with increasing oxidation and the addition of oxygen containing substituents. Unfortunately this often results in the production of carboxylic, sulfinic and sulfonic acids. These small extremely polar compounds are no longer amendable to extraction and analytical procedures commonly utilized in the study of biodegradation pathways.

A second trend is the formation of larger structures through oxidation and condensation reaction. Examples of this include the formation of thioindigo,

benzonaphthothiophenes, and disulfides from 2-mercaptophenylglyoxylate. Often metabolites can be observed to be inseparable from the void volume during reverse phase HPLC and also not come through normal phase columns or be susceptible to routine GC protocols. One of the main obstacles to this dissertation was the necessity for the creation of new protocols and procedures to detect, identify and quantify these transformation products. One example of this was the identification of the disulfide compounds formed from benzothiophene-2,3-dione. This was accomplished by developing higher temperature GC protocols coupled to stringent sample preparation protocols including avoiding drying agents, choice of solvents and diazomethane derivatization. Unfortunately, observation of HPLC profiles sometimes revealed other unidentified metabolites which could not be separated from the aqueous phase for analysis. These metabolites were also too polar for HPLC mass spectrometry analysis due to their polarity which required mobile phases with low pH and high buffering capacity which were unsuitable for HPLC mass spectrometry. Perhaps someday in the near future when alternate techniques such as solid-phase micro-extraction are developed to the extent that extremely polar small metabolites can be extracted, some of these metabolites will be identified.

Chapters 2 and 3 of this dissertation focused on the biodegradation of benzothiophene sulfone, 5-fluorobenzothiophene sulfone, and methylbenzothiophene sulfones. There have been indications that there could possibly be more than one attack pathway utilized by strain DB1 for the biodegradation of the sulfones. Further investigations using dimethylbenzothiophene sulfones could perhaps be used to clearly demonstrate the two different attack pathways. The use of dimethylbenzothiophene sulfones, methylfluorobenzothiophene sulfones and other similar compounds is mostly limited by the availability of these compounds. Most likely, these types of studies would require chemical synthesis of these substrates. The chances of finding further biodegradation metabolites of benzothiophene sulfone biodegradation, past the dihydrohydroxybenzothiophene step would also be enhanced by studying increasingly substituted benzothiophene sulfones.

During the course of this thesis, there were three attempts to isolate bacteria capable of utilizing benzothiophene sulfones as sole carbon, energy and sulfur sources.

Unfortunately, all failed except for the initial effort which yielded strain DB1. It is interesting that the benzothiophene sulfones appear to be somewhat recalcitrant given their oxidized nature. To further understand the biodegradation of the benzothiophene sulfones, it may be beneficial to isolate other bacterial strains capable of either co-metabolizing benzothiophene sulfones or utilizing them as sole carbon, energy and sulfur sources. The use of strain DB1 for biodegradation studies has more than its share of complications. Strain DB1 is an aggregating filamentous bacterium and thus simple microbiological techniques such as pelleting of cells, following optical density for cell growth, obtaining biomass concentrations, and obtaining reproducible inoculum are next to impossible to apply to this strain. Strain DB1 also has the complicating characteristic of having a long lag period followed by a rapid log phase in which the strain is very efficient at mineralizing the sulfones. These factors combine to make identification of biodegradation metabolites very difficult. The use of a different biodegrading strain may make identification of biodegradation metabolites much easier.

The identification of novel benzothiophene sulfone biodegradation and condensation products provides further substrates for biodegradation studies. If the dihydrohydroxybenzothiophene sulfones can be synthesized, it would be of interest to subject them to biodegradation studies by strain DB1 or other bacterial strains to try to further characterize this biodegradation pathway. Having large concentrations of this known metabolite may increase the chances of observing further biodegradation products.

The recognition of benzonaphthothiophene sulfone as a condensation product of benzothiophene sulfone adds more evidence to the argument that the whole class of benzonapthothiophenes should be incorporated into biodegradation studies. The Diels-Alder condensation producing these large products is known to be abiotic and thus the presence of benzothiophene sulfoxides and sulfones in the environment would suggest that these large benzonaphthothiophenes would also be present as environmental contaminants. Due to their large size and hydrophobic nature, these compounds would be expected to be rather recalcitrant, but due to their low aqueous solubility, not very mobile in the underground water tables and thus would accumulate.

One relatively unexplored metabolite observed in the biodegradation of the condensed thiophenes in general is the hydroxy, and methanol substituted

benzothiophenes produced by dioxygenase attack of the aromatic rings or the methyl substituents (reviewed by Kropp *et al.* 1998). It is apparent that these hydroxy-substituted metabolites may be a step in the biodegradative pathways of the parent condensed thiophenes. If some of these hydroxylated intermediates can be synthesized, they would serve as excellent informative biodegradation substrates, hopefully allowing the elucidation of the complete biodegradation pathways.

In chapter 4 of this dissertation, the chemical properties and biodegradation of HFBT was investigated. One obvious conclusion of this study is that HFBT would not be expected to be a stable accumulating metabolite in the environment. For this reason future studies should be focused at investigating thioindigo which was shown to be the major accumulating HFBT condensation product. Thioindigo is commercially available making it an easy substrate to obtain for biodegradation studies. A recent review of the literature indicated there have been no investigations into the biodegradability of thioindigo. One other possibility for a biodegradation substrate leading from this work would be 2-mercaptophenylglyoxalate (or benzothiophene-2,3-dione) based on the fact that the concentration of this compound was increased during biodegradation studies with HFBT which may mean the 2,3-dione is the next product in the HFBT biodegradation pathway. The biodegradation of the 2,3-dione was the topic of Chapter 5 of this thesis.

Benzothiophene-2,3-dione was shown to be unstable in the open ring form, or 2-mercaptophenylglyoxylate at neutral pH. It was observed that the 2-mercaptophenylglyoxylate underwent an abiotic oxidation to form the corresponding disulfide. Subsequent decarbonylations were observed resulting in 2,2'-dithiosalicylic acid. The fact that this oxidation and subsequent decarbonylation occurred so rapidly indicated that the 2,3-dione would not be expected to be very stable in the environment. Future biodegradation investigations would find more success and more applicability if they focused on the biodegradation of the disulfides including and most likely the 2,2'-dithiosalicylic acid. This dissertation clearly demonstrated that the 2,2'-dithiosalicylic acid is subject to further biodegradation, but these studies were restricted due to time limitations. Further biodegradation studies on the 2,2'-dithiosalicylic acid should focus on metabolite identification and on the mineralization of the substrate. One area of future investigations that would strengthen this area of knowledge would be to investigate the

chemical properties and biodegradability of methyl-, and dimethyl substituted benzothiophene-2,3-diones as well as any formed disulfide products. The 5- and 7-methylbenzothiophene-2,3-diones have been previously synthesized in the laboratory and are available for further study.

This thesis has focused primarily on the biodegradation of known metabolites of condensed thiophenes. Although it was originally hypothesized that further biodegradation of these metabolites would result in smaller further oxidized polar metabolites, surprisingly the recurring theme of this dissertation is that larger oxidized and condensed metabolites were formed. Further studies are needed to accurately determine the percentage of the primary metabolites that end up as larger recalcitrant products. This dissertation should serve as a foundation from which further studies can investigate the role that formation of these larger products has on the overall balance of carbon and sulfur in biodegradative processes.

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APPENDIX A. OXIDATION OF CARBAZOLE, N-ETHYLCARBAZOLE, FLUORENE, AND DIBENZOTHIOHENE BY THE LACCASE OF *CORIOLOPSIS GALLICA* UAMH 8260*

A.1 INTRODUCTION

The polycyclic heterocycles carbazole, dibenzothiophene and dibenzofuran are all analogs of the polycyclic aromatic hydrocarbon fluorene but containing nitrogen, sulfur and oxygen respectively as the bridging atom between the two aromatic rings. All four compounds are found in coal tar, creosote, and some sources of crude oil, and are released into the biosphere during incomplete combustion of these sources. These polycyclic compounds are relatively recalcitrant to microbial attack, though they are known to be oxidized by bacteria and a considerable body of experimental data has accumulated during the past 20 years (Bressler and Fedorak 2000). In the biosphere they are both ecological and public health problems, and their presence in crude oil streams both reduces the value of the refined product and produces greenhouse gases.

Bacterial oxidation of these compounds has been shown to follow three distinct patterns. These include angular dioxygenase attack adjacent to the bridging atom, dioxygenase attack of the aromatic rings or monoxygenation attack at the methylene position in carbazole or at the sulfur atom of dibenzothiophene (Bressler and Fedorak 2000).

White rot fungi have been studied for their ability to initiate degradation of recalcitrant organo-pollutants such as polycyclic aromatic hydrocarbons (PAHs, Bogan and Lamar 1996; Majcherczyk *et al.* 1998; Pickard *et al.* 1999), chlorinated phenols (Ruckenstein and Wang 1994), PCBs (Beaudette *et al.* 1998, 2000; Sasek *et al.* 1993), dioxins (Takada *et al.* 1996), pesticides (Kullman and Matsumura 1996), explosives (Gorontzy *et al.* 1994), dichloroaniline (Arjmand and Sandermann 1985) and dyes (Kirby *et al.*, 1995; Shin *et al.*, 1997; Reyes *et al.* 1999). In this study, purified laccase from the

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white rot fungus *Coriolopsis gallica* was tested for its ability to oxidize the PAH fluorene and five related polycyclic heterocycles.

A.2 MATERIALS AND METHODS

A.2.1 Enzyme and chemicals

Purified laccase (Laccase, EC 1.10.3.2) was obtained from *Coriolopsis gallica* UAMH8260, as reported previously (Rodriguez *et al,* 1999). Acetonitrile and methylene chloride (HPLC grade) were from Fisher Chemicals (Fair Lawn, N.J.) and 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) diammonium salt and 1-hydroxybenzotriazole (HBT) were purchased from Sigma (St Louis, Mo). The substrates benzothiophene, dibenzofuran, fluorene, and carbazole were from Aldrich Chemical Company, Inc. (Milwaukee, Wis.); dibenzothiophene was obtained from Fluka Chemika (Buch, Switzerland); and N-ethylcarbazole was from Pfaltz & Bauer, Inc. (Stamford, Conn). Dibenzothiophene sulfoxide was purchased from IGN pharmaceuticals, Inc. (Plainview, N.Y.), and 9-fluorenone was obtained from Aldrich.

A.2.2 Laccase assay.

Laccase activity was determined by ABTS oxidation (Woolfenden and Wilson 1982) in a reaction containing 1 mM ABTS in 0.1 M sodium acetate buffer pH 4.5 and 5 to 50 μL enzyme sample. Oxidation was followed at 436 nm and one unit of activity was defined as 1 μmol ABTS oxidized/min (ε_{436}=29,300 $M^{-1}cm^{-1}$).

A.2.3 Substrate oxidation.

Reaction mixtures contained the individual substrate (20 μM), ABTS (1 mM), and HBT (1 mM) in 0.1 M acetate buffer, pH 4.5 containing 15% acetonitrile with 0.01 to 5 units *C. gallica* laccase in a 100 μL reaction volume. In 15% acetonitrile, *C. gallica* laccase exhibited 91% of the activity in buffer alone. The assay was started by the addition of enzyme and terminated by addition of 100 μL acetonitrile. Boiled enzyme controls had no activity. After centrifugation, 50 μL samples were analyzed by HPLC using a Waters system and a Brownlee Spherisorb-10 μm RP18 column (Applied Biosystems) with isocratic elution using 60:40 acetonitrile:water. Substrates were

monitored by UV absorbance at the following wavelengths: dibenzothiophene 235 nm, dibenzofuran 280 nm, fluorene 263 nm, and carbazole 235 nm. Oxidation was calculated from reduction in peak area and compared to a standard curve.

To obtain enough products for identification on gas chromatography-mass spectrometry (GC-MS), 10-mL reaction mixtures containing 20 μM substrate were treated with laccase (5 units), 1 mM ABTS and 1 mM HBT, except for carbazole where 1 unit of laccase was used. In the cases of dibenzothiophene and fluorene a total reaction volume of 50 mL was used with 25 units of laccase. After an 18-h reaction, the mixtures were acidified and extracted three times with equal volumes of methylene chloride. The extracts were combined, dried over anhydrous sodium sulfate and concentrated under nitrogen prior to analysis on a Hewlett Packard 5890 series II gas chromatograph with a 5970 series mass selective detector (GC-MS). The chemical structure of the oxidation products was determined by comparing their retention times and mass spectra with those of commercially available chemical standards. After GC-MS analysis of initial methylene chloride extracts, all extracts were subsequently derivatized with diazomethane freshly prepared using a millimole-size MNNG apparatus (Aldrich) and subsequently analyzed by GC-MS.

A.2.4 Protein determination.

The protein content of laccase preparations was determined by the Bradford protein assay (BioRad, Mississauga, ON)

A.3 RESULTS AND DISCUSSION

Using small scale reaction mixtures (0.1 mL) and 5 laccase units for 1 h, the sensitivity of the polycyclic heterocycles to oxidation by laccase was shown to be carbazole (100% loss) > fluorene (75%) > dibenzothiophene (60%). Carbazole was found to be about 100 times more sensitive to laccase oxidation than fluorene and dibenzothiophene. No reaction occurred in the absence of mediating substrates over this period. Benzothiophene and dibenzofuran showed no detectable oxidation in the presence of laccase and mediating substrates.

The oxidation products of dibenzothiophene and fluorene were identified by GC-MS analyses of methylene chloride extracts of the reaction mixtures and of authentic standards. The product of dibenzothiophene oxidation was dibenzothiophene sulfoxide (Figure A.1). The oxidation product had the same retention time (Figure A.1a and A.1b) and the same mass spectrum (Figure A.1c and A.1d) as the authentic standard.

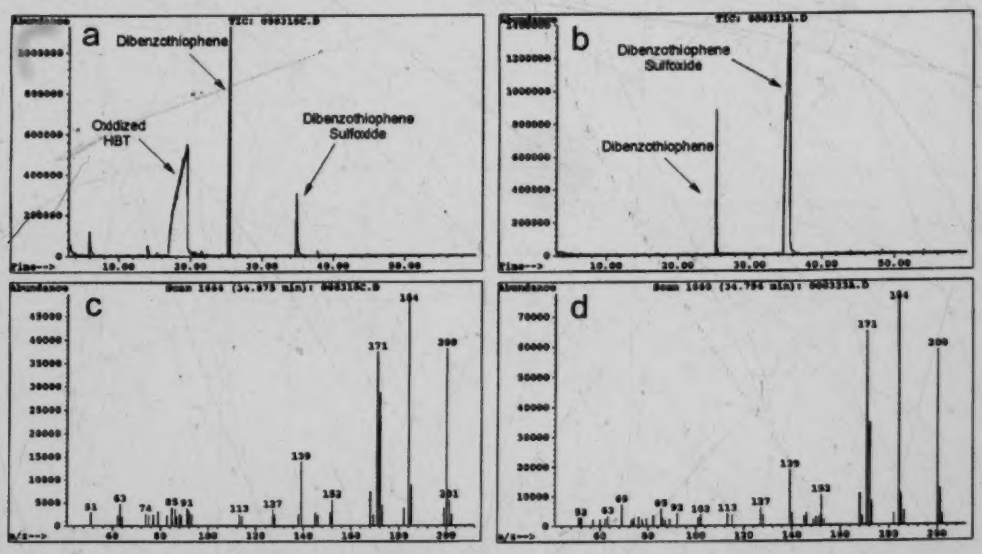

Figure A.1 GC-MS analyses of the extract of a reaction mixture that contained dibenzothiophene (a,c) and of an authentic standard of dibenzothiophene sulfoxide (b,d).

Similarly, the oxidation product of fluorene had the same retention time (Figure A.2a and A.2b) and the same mass spectrum (Figure A.2c and A.2d) as the authentic standard of 9-fluorenone. Analysis of the extracts after attempts to methylate with diazomethane showed no additional products.

Extracts of reaction mixtures that contained only HBT and laccase gave the same broad peak with retention time of about 19 min that was found in Figures A.1a and A.2a. Thus this peak was attributed to the formation of an oxidation product of HBT. The dibenzothiophene in Figure. A.1b may have been a contaminant in the commercially available dibenzothiophene sulfoxide, or it may have been caused by thermal decomposition of the sulfoxide in the GC injection port (Vignier *et al.* 1983).

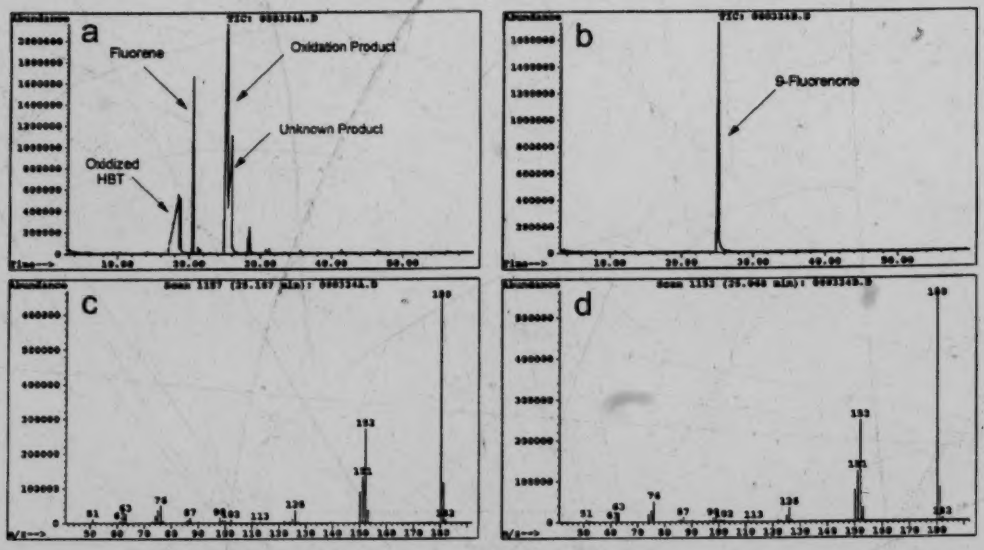

Figure A.2 GC-MS analyses of the extract of a reaction mixture that contained fluorene (a,c) and of an authentic standard of 9-fluorenone (b,d)

GC-MS analysis of extracts from the resulting mixtures that had contained carbazole showed no oxidation product or residual carbazole. Treating the extracts with diazomethane failed to yield any peaks on GC-MS analysis.

To help determine if the nitrogen atom of carbazole was the site of oxidative attack, N-ethylcarbazole was also tested as a substrate. N-Ethylcarbazole was completely removed from the reaction mixture, again leaving no detectable products using either direct extraction or extraction with derivatization. In case an oxidized product of carbazole was reacting with either other oxidized carbazole molecules or with oxidized forms of the accelerating substrates, control reactions were conducted lacking one or both of the accelerators. If both accelerators were removed, no oxidation of carbazole was detected even after one week of incubation. If either accelerator was included, the carbazole was efficiently oxidized after an overnight incubation, but oxidized products remained undetectable. Since laccase oxidation employs a radical oxidation mechanism (Gianfreda *et al.* 1999) it may be that the product of carbazole oxidation becomes sufficiently condensed and polymerized so as to be undetectable by the methods we used.

Electrochemical oxidation of carbazole produces dimers and possibly polymers (Ambrose *et al.* 1975). On no occasion were precipitates observed in our reaction mixtures.

The results from this study are summarized in Figure A.3, and the susceptibility to oxidation by *C. gallica* laccase appears to be related to the ionization potential (IP) of the compound being tested (Kersten *et al.* 1990). Compounds with IPs greater than 8.52 were not oxidized under these conditions. However this is a small selection of potential substrates and studies with larger groups of PAHs have shown only a limited relationship between laccase activity and the IP of PAHs (Majcherczyk *et al.* 1998; Pickard *et al.* 1999).

That dibenzothiophene and fluorene are both subject to oxidation forming dibenzothiophene sulfoxide and 9-fluorenone respectively is reminiscent of oxidations found in many bacterial systems (for review see Bressler and Fedorak 2000). In the bacterial system, susceptibility to angular dioxygenase attack appears dependent on the electronegativity of the atom bridging the two aromatic rings. However in the fungal laccase system, susceptibility to oxidation appears more closely related to the IP of the substrate.

This is the first report of the oxidation of dibenzothiophene, carbazole and N-ethyl carbazole by a fungal laccase.

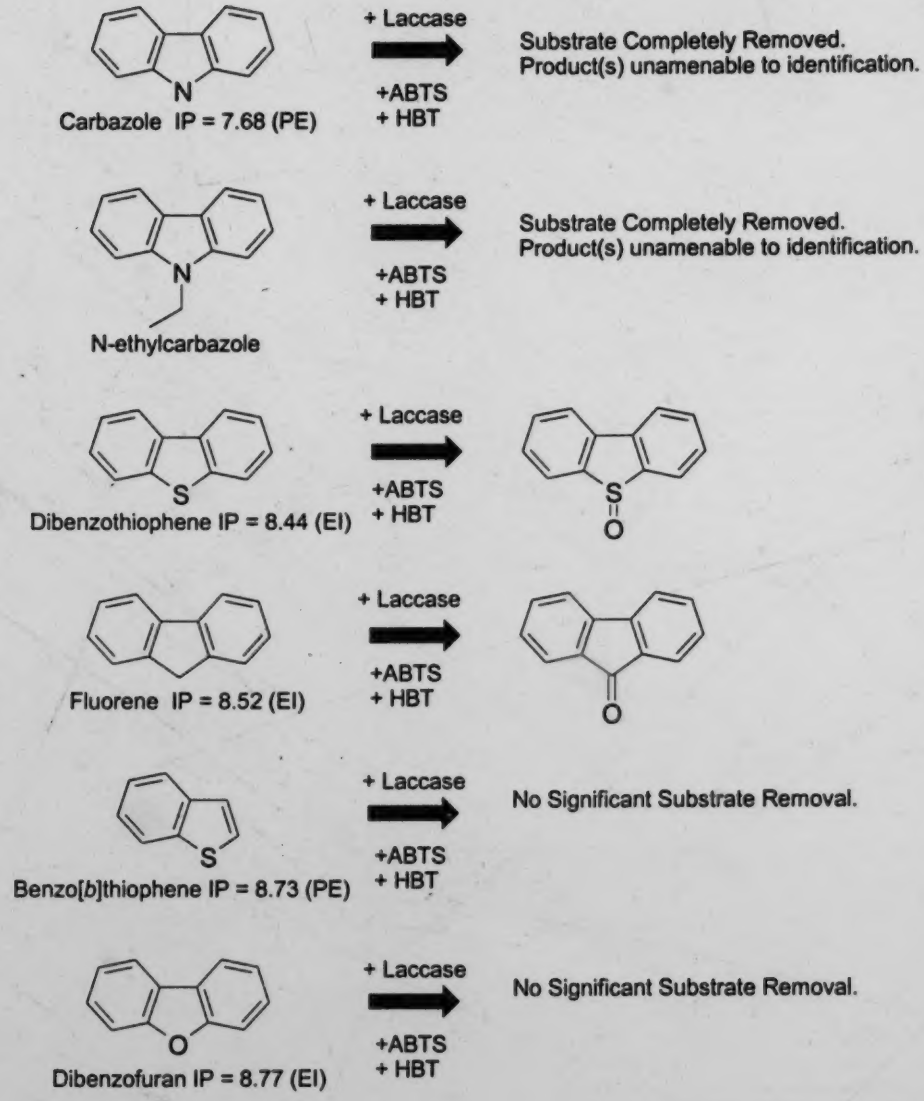

Figure A.3 Summary of results with various substrates in reaction mixtures with laccase. IP values reported by Levin and Lias (1982), were determined by photoelectron spectroscopy (PE) or electron impact (EI). No IP could be found for N-ethylcarbazole.

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